Understanding the molecular and biochemical basis of insecticide selectivity against solitary bee pollinators

Submitted by Katherine Beadle to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, September 2018

Abstract

Certain eusocial bee pollinators have been found to exhibit profound differences in their sensitivity to different chemical insecticides within the same class (e.g. the *N*-nitroguanidine neonicotinoid imidacloprid and the *N*-cyanoamidine thiacloprid). Recent work on honey bees and bumblebees has shown that this variation in sensitivity is due, at least in part, to differences in the capacity of cytochromes P450 belonging to the CYP9Q subfamily to detoxify different insecticides. The solitary red mason bee, *Osmia bicornis*, is the most economically important solitary pollinator in Europe, yet its sensitivity to insecticides is not well characterised. Topical insecticide bioassays revealed that like honey bees and bumblebees, *O. bicornis* exhibits significant differences in sensitivity to insecticides within the same class, demonstrated by a >2,000-fold difference in sensitivity to imidacloprid and thiacloprid. Radioligand competition assays revealed no significant differences in the binding affinity of imidacloprid and thiacloprid to nicotinic acetylcholine receptors (nAChRs) isolated from head membrane preparations, demonstrating that differences in the binding affinity of imidacloprid and thiacloprid for nAChRs does not explain the marked variation in bee sensitivity to these compounds. Furthermore, cuticular penetration assays revealed no difference in the rate of penetration of these insecticides. The differential sensitivity observed during toxicity bioassays was found to be greatly suppressed on addition of the P450 inhibitor, PBO, prior to thiacloprid application but not imidacloprid application, suggesting that P450s play a role in determining the sensitivity of *O. bicornis* to neonicotinoid insecticides.

Sequencing of the transcriptome and genome of *O. bicornis* along with the annotation of other bee genomes revealed that *O. bicornis* and most other solitary bee species lack the *cyp9q* subfamily. Subsequently, the most closely related *O. bicornis* P450s to this subfamily were selected as potential thiacloprid-detoxifying orthologs for further characterisation. Kinetic studies revealed that six of the recombinantly expressed P450s were able to metabolise

both imidacloprid and thiacloprid. However, all of the catalytically active P450s displayed a greater affinity for thiacloprid compared to imidacloprid, which could enable its rapid metabolism before any detrimental effects can occur, explaining, at least in part, its comparatively low toxicity. The most effective neonicotinoid metaboliser was found to be CYP9BU1, which also has the capacity to metabolise a number of other insecticides, suggesting that it may be a key detoxification enzyme of *O. bicornis. O. bicornis* microsomes displayed substantial ability to metabolise the secondary plant metabolite nicotine and incubation of recombinant P450s with nicotine identified CYP6AQ55 as a major nicotine metabolising enzyme. Taken together these findings illustrate that the CYPome of *O. bicornis* contains P450s that can metabolise both natural and synthetic insecticides.

Insecticide-metabolising P450s were found to be highly expressed in the Malpighian tubules, a primary site of xenobiotic detoxification, and the brain of *O. bicornis*, which contains high concentrations of nAChRs. Exposure of *O. bicornis* to sublethal doses of either imidacloprid or thiacloprid did not induce the expression of any P450s, suggesting constitutive expression of insecticide-metabolising P450s provides protective effects.

The knowledge generated in this thesis can be leveraged to help avoid negative insecticide impacts on this important solitary bee pollinator and provide tools to aid the design of bee-safe insecticides.

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I would like to dedicate this thesis firstly to the Beadle clan- Hazel, Andrew, Sarah, Will, Richard, James, Jess, Poppy, Tigger and Nala. Thank you for supporting me and showing your interest through bee-themed gifts. Mum- thanks for finding and encouraging me to apply to this PhD. Dad- hopefully there are 'some big words' in here to make you proud.

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Enjoy!

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List of Abbreviations

AA	Amino acid
ACh	Acetylcholine
BFC	7-Benzyloxy-4-(trifluoromethyl)-coumarin
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BOMFC	7-(benzyloxymethoxy)-4-trifluoromethylcoumarin
BOMR	(benzyloxymethoxy)resorufin
bp	Base pairs
BUSCO	Benchmarking universal single-copy orthologues
C	Celsius
CCE	Carboxylesterase
cDNA	Complementary deoxyribonucleic acid
CHC	Cuticular hydrocarbons
CPR	Cytochrome P450 reductase
CYP	Cytochrome P450
CYPome	Cytochrome P450 complement
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
dpm	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
EFC	7-ethoxy-4-trifluoro-methylcoumarin
EFSA	European food safety authority
EPA	U.S. environmental protection agency
FBS	Fetal bovine serum
fpkm	fragments per kilobase of transcript per million mapped reads
g	Gram
GC clamp	The presence of G/C bases within last five bases from the 3' end of primers
GC content	Guanine-cytosine content
g-force	Relative centrifugation force
GO	Gene ontology

GST	Glutathione-S-transferase
HPLC-MS	High performance liquid chromatography mass spectroscopy
IC_{50}	Concentration of unlabelled ligand displacing 50% of [3H]imidacloprid from its binding site
IRAC	Insecticide resistance action committee
K_m	Substrate concentration needed to reach 1/2 of the maximum velocity (V_{max}) in a reaction
LB	Lysogeny broth
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Dose required to cause 50% mortality in test subjects
LGIC	Ligand-gated ion channel
MEGA	Molecular evolutionary genetic analysis
mg	Miligram
mM	Milimolar
MoA	Mode of action
nAChR	Nicotinic acetylchloine receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National center for biotechnology information
ng	Nanogram
OECD	Organisation for Economic Co-operation and Development
OP	Organophosphate
ORF	Open reading frame
P450	Cytochrome P450
PBO	Piperonyl butoxide
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
PHYML	Phylogenetic interferences using maximum likelihood
POLO	Probit or Logit analysis
PolyA tail	Poly adenylated tail
qPCR	Quantitative real-time polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR	Resistance ratio

rSAP	Recombinant shrimp alkaline phosphatase
SAR	Structure-activity-relationship
SE	Standard error
SPM	Secondary plant metabolites
SOC	Super optimal culture
TAE	Tris-acetate-EDTA
TEPP	Tetraethylpyrophosphate
TGAC	The genomic analysis centre
T_m	Primer melting temperautre
Trisophila	Transgenic fly line containing three genes of interest
UAS	Upstream activating sequence
µM	Micromolar
UPGMA	Unweighted pair group method with arithmetic means
V	Voltage
VGSC	Voltage-gated sodium channel
V_{max}	Maximum velocity of a reaction

Chapter 1: General introduction

1.1 Food security and the importance of pollinators

Food security is said to exist 'when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meet their dietary needs and food preferences for an active and healthy life' (United Nations, 2002). Food security is a multi-dimensional issue, and therefore difficult to measure. Several indices of measurement are currently used including The Food Insecurity Experience Scale (FIES), which is a model-based scale consisting of eight questions about people's access to adequate food (Cafiero et al., 2018). One major challenge to achieving food security is the ever-rising human population, which is expected to reach anywhere between 8.1 and 10.6 billion by 2050. It is predicted that to meet the resultant increasing demands food production will need to increase by 70% (United Nations, 2011).

Pollinators are a vital component of food security, currently providing essential pollination services to around three-quarters of crops, with the demand for insect pollination continuing to rise (Breeze et al., 2011). Recent figures suggest that the contribution of pollinated crops to the UK crop market is £430 million per annum (8%) (UK National Ecosystem Assessment Technical Report, 2014) and is estimated to be between €153 billion (Gallai et al., 2009) and $361 billion worldwide (Lautenbach et al., 2012). Currently, most crops rely on a small number of managed honey bees for pollination, although the risks of relying on a single species of bee are becoming increasingly obvious, and there is growing awareness of the importance of solitary bees as pollinators (Gruber *et al.*, 2011). Greater functional pollinator diversity has been found to increase crop yield (Fründ *et al.*, 2013; Hoehn e*t al.,* 2008) and in some cases solitary bees have been found to be more efficient pollinators compared to honey bees. For example, a study by Woodcock *et al*. (2013) found that the visitation of solitary bees to oilseed rape flowers was twice as likely to result in pollen transfer to the stigma compared to honey bees, although as honey bees numerically outweighed the solitary bees this may not have been of importance. It has also been found that the presence of wild bees can enhance the effectiveness of

honey bee flower visitation, with honey bees being more likely to move to a female flower from a male flower after interacting with a wild bee (Greenleaf and Kremen, 2006). *Osmia bicornis* is one such solitary bee species used commercially for the pollination of tree fruit crops such as almond, peach, apricot, plum, cherry, apple and pear (Sedivy and Dorn, 2014). Recently, this species has become commercially available in Poland, and is used as pollinator from February until July (Giejdasz, 2016).

Not only are pollinators of economic importance but are also depended upon by a number of wild plant species for reproduction. The parallel decline of pollinators and insect-pollinated plants demonstrated by Biesmeijer et al. (2006) highlights the importance of this symbiotic relationship. In turn, these insect-pollinated plants provide food and shelter to numerous organisms within a range of ecosystems (Vanbergen, 2013).

1.2 *Osmia bicornis*

There are over 20,000 described species of bee worldwide and around 90% of these are solitary (Linsley, 1958; Michener, 1974). A solitary bee can be defined as a species of bee that does not live in colonies, and emerging adult bees must locate food without any guidance from older adults (Dobson et al., 2012). Among the larger genera of solitary bees is *Osmia* with over 100 species described (Linsley, 1958). The Red Mason Bee, *Osmia bicornis* or *Osmia rufa* (Linnaeus, 1758; Hymenoptera: Megachilidae), is one of the most common species, found in England, Scotland, Wales, Ireland, mainland Europe, Sweden, Norway, North Africa, Georgia, Turkey and Iran (Raw, 1972).

1.2.1 The life cycle of *O. bicornis*

1.2.1.1 Emergence

The active season of the univoltine *O. bicornis* begins in mid-April when temperatures begin to rise, triggering the activity of the bees. The males become active first, using their mandibles to chew their way out of their cocoons

and subsequently through their cell partitions. Once emerged, the males will tend to loiter around the nesting area, waiting for the females to emerge. The emergence time of females can range between a few days and a few weeks after the males emerge, and can continue until late-June (Seidelmann, 1999b). Occasionally a bee will chew through its cell partition only to find a still dormant bee in the next brood cell obstructing its emergence path. The emerging bee will chew through the cocoon of the dormant bee and nip its abdomen to wake it up. The bee will continue to nip the dormant bee until it also emerges from its cocoon. Then together they will chew their way through the next partition in sequence until they are able to leave the nest (Raw, 1972).

Adult male and female *O. bicornis* can be sexed according to the differing colour of the clypeus (females: black, males: white; figure 1) (Seidelmann, 2014). Furthermore, similar to other solitary bee species, *O. bicornis* exhibits high levels of sexual size dimorphism, with the female body weight being around 1.6 times greater than the male body weight (Seidelmann et al., 2010; Seidelmann, 2014; Sandrock et al., 2014). On average, adult male *O. bicornis* have a lifespan of 4 to 6 weeks, and females 8 to 10 weeks (Fliskiewicz et al., 2015).

1.2.1.2 Copulation

The mating system of this species can be defined as scramble competition polygyny (Seidelmann, 1999b). The males are polygynous, attempting to mate with as many females as possible in order to maximise their reproductive success. The nest sites are generally highly scattered and so the mating success of males seems to be highly dependent on a males ability to locate a female rather than on other factors such as body size (Seidelmann, 1999b). As the probability of mating is low and the competition for females is high, male *O. bicornis* have evolved several mechanisms to ensure their paternity. For example, males are able to behaviourally induce unreceptivity to mating in females by performing a post-copulatory display. Males have also been observed using a mating plug containing spermatozoa as a reinsurance measure. As a result of these mechanisms female *O. bicornis* tend to mate only once (monandry) (Seidelmann, 2014), exhibiting a preference for more closely

related intermediately-sized males that display longer precopulatory vibration duration (Conrad et al., 2010).

1.2.1.3 Construction of nests and egg laying

As the only contribution of males to their progeny is their sperm, the reproductive output is solely dependent on the females foraging and nest-building abilities (Seidelmann, 1999b). After mating, females will complete an orientation flight and find a suitable nest site, often nesting gregariously and close to the site from which they emerged (Linsley, 1958). Once they have found an ideal nesting site they will then commence the construction of nests, utilising pre-existing holes such as those in brick walls or those made by boring beetles. Within these holes the females create a series of cylindrical brood cells. Within each brood cell the female provides a food package consisting of pollen and nectar kneaded into a paste (Raw, 1972).

O. bicornis is a polylectic bee species, visiting up to 140 different plant species (e.g. *Ranunculus acris* and *Sinapis arvensis*), although analysis of brood cell provisions suggests that females tend to restrict pollen collecting to a few taxa at a time, resulting in brood cells typically containing pollen from mainly one or two plant species/ pollen families (Dobson et al., 2012; Radmacher and Strohm, 2010; Sedivy et al., 2011).

The food package provided by the female is a desirable resource for parasitoids, and subsequently brood cell parasitism can occur during the provisioning of cells. There are three main parasites of *O. bicornis*; Krombein's hairy-footed mite *Chaetodactylus osmiae*, the small drosophilid fly *Cacoxenus indagator*, and the bombylid fly *Anthrax anthrax*, which loiter outside of the nest entrance and lay their eggs whilst the female is absent (Seidelmann, 2006).

Once a female has gathered enough food she lays a single egg on top and seals the cell with a combination of mud and salivary secretions (Keller et al., 2013). She repeats this many times, sequentially constructing a row of up to 31 cells (Dobson et al., 2012; Giejdasz, 2016).

Female *O. bicornis* have a high level of control over both the body size and the sex of each offspring (Bosch, 2008; Seidelmann, 2014). The body size of the offspring is dependent on the amount of provisions allocated to each brood cell, with female progeny occupying larger brood cells containing more food (Radmacher and Strohm, 2010; Rooijakkers and Sommeijer, 2009). The increased allocation of food means that female offspring are more costly to produce and thus the offspring sex ratio can be used as a measure of the condition of the nesting female (Sandrock et al., 2014). The sex of each offspring is controlled by the release of sperm from the spermathecal, with fertilised eggs resulting in diploid females and unfertilised eggs resulting in haploid males (Bosch, 2008; Seidelmann et al., 2010). This genetic system is known as haplodiploidy and is common in Hymenoptera (Dmochowska-Ślęzak et al., 2015). Female-destined eggs are laid in brood cells towards the rear of the nest and male-destined eggs towards the nest entrance, allowing the males to emerge first and await copulation.

At the entrance of the nest the female constructs an empty cell known as a vestibular which has a partition that is 1.5-2 times thicker than a regular partition (Bosch, 2008). The function of the vestibular cell is thought to be to protect progeny against fluctuating external environmental conditions (Seidelmann, 1999a). Following the vestibular cell is the terminal plug which seals the nest (Raw, 1972).

1.2.1.4 Larval development

The eggs hatch approximately one week after being laid and the larvae consume the food provided (Keller et al., 2013). The larvae then begin to spin cocoons, pupating around 50 days post-hatching (see figure 2). The imago then enter obligatory diapause (November-January) and postdiapause quiescence (January-emergence) which allows them to survive over winter (Wasielewski et al., 2011).

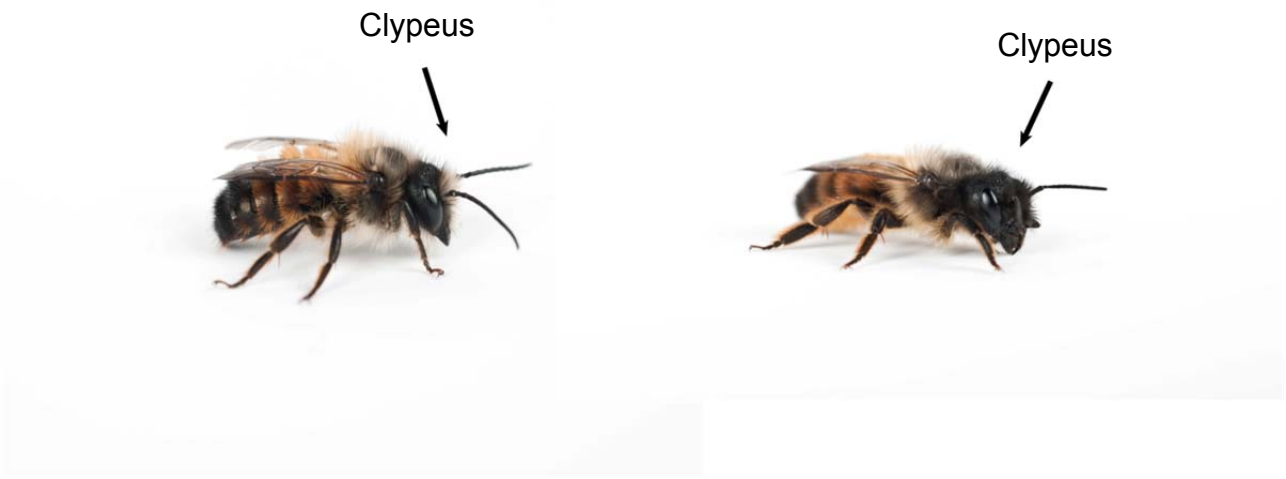


Figure 1. Male (left) and female (right) adult *O. bicornis*.

Figure 2. Adult male *O. bicornis* emerging from cocoon.

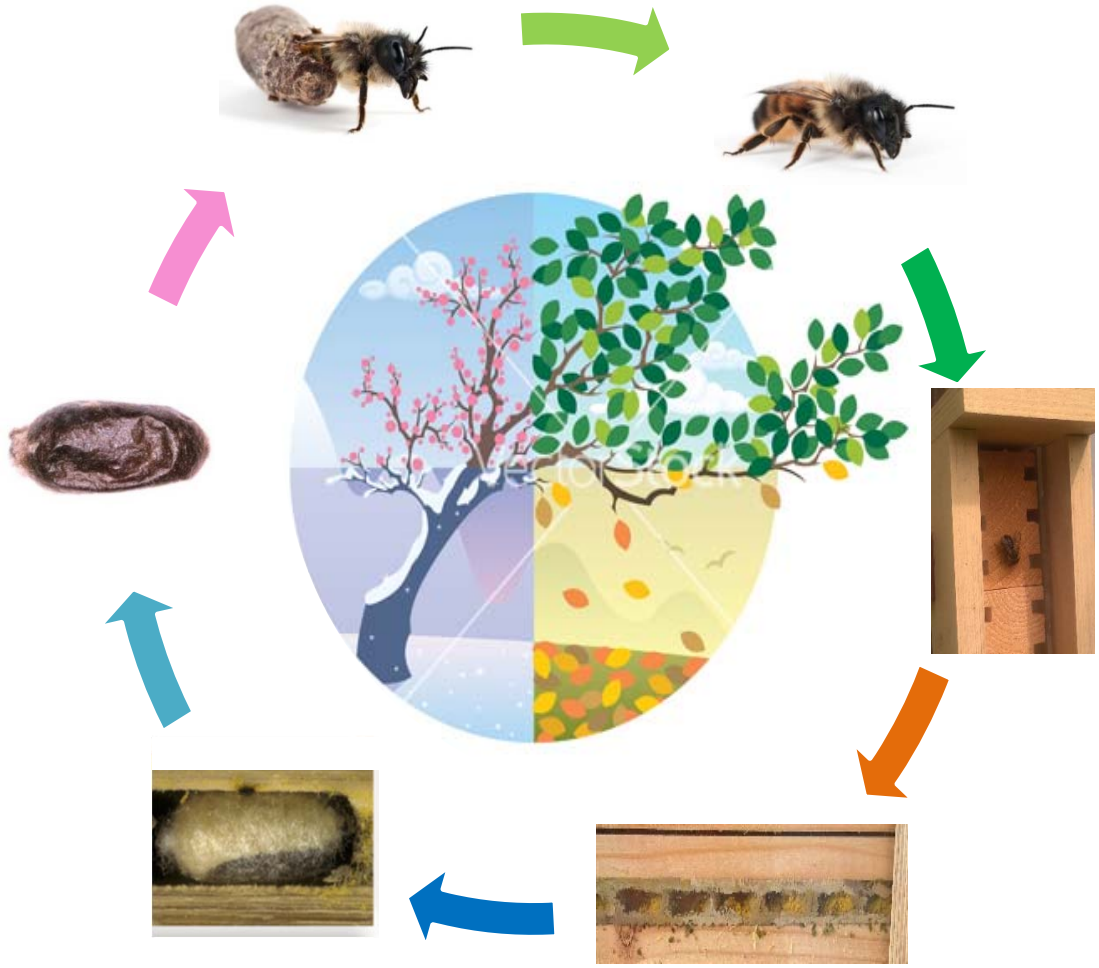


Figure 3. Diagram of the life cycle of *O. bicornis* shown as a sequence of events; 1) emergence, 2) copulation, 3) construction of nests and egg laying, 4) consumption of food package, 5) obligatory diapause, 6) postdiapause quiescence.

1.3 Pollinator decline and factors affecting bee health

There is accumulating evidence that both the abundance and diversity of pollinators are in decline. To date, this decline has been reported in Europe (Biesmeijer et al., 2006; Fitzpatrick et al., 2007; Ollerton et al., 2014; Potts et al., 2010), North America (Cameron et al., 2011; Colla and Packer, 2008) and Asia (Williams and Osborne, 2009). The reasons for this decline remain elusive, but there are a number of factors that could affect bee heath, some of which are

outlined below.

1.3.1 Changing weather patterns

Changing weather patterns can impact pollinators both directly and indirectly. Directly, sub-optimal temperatures have been found to influence performance (Tautz et al., 2003), development (Wang et al., 2016), and the life span (Bosch et al., 2000) of a number of bee species. Indirectly, climate changes can cause a shift in plant-pollinator ranges, and alter the synchrony of plant flowering and the active period of pollinators (Morton and Rafferty, 2017). Furthermore, numerous studies have documented the detrimental effects that changing weather conditions can have on crop yield per hectare (Lesk et al., 2016; Rosenzweig et al., 2002; Schlenker and Roberts, 2009), which as well as being a vital food source for many pollinators, may also result in an increased volume of land required for crop productivity and subsequently a decrease in natural habitat.

Some studies have noted that bee pollinators have great adaptive potential to changing climatic conditions. In particular *Apis mellifera,* which is found around the globe in a great variety of climates. Indeed, in the hot climate of Arizona honey bees are able to survive by using large quantities of water to regulate the brood temperature (Le Conte and Navajas, 2008; Rader et al., 2013).

1.3.2 Pests and diseases

Bees are susceptible to a great number of parasites which can significantly weaken or kill colonies when reaching epidemic infestation levels (Otti and Schmid-Hempel, 2008). One of the most well studied parasites is the ectoparasitic mite *Varroa destructor* that infects honey bees. Originally restricted to the Eastern European honey bee *Apis cerana*, a host-shift to *A. mellifera* in the last century played a central role in its spread worldwide, with the exception of Australia which remains *Varroa*-free (Rosenkranz et al., 2010). This mite feeds on the developing larvae and adults of colonies, transmitting viruses such as deformed wing virus, which has been found to be a significant

contributor of the syndrome Colony Collapse Disorder (CCD) (vanEngelsdorp et al., 2009). Solitary bees are also susceptible to a number of parasites such as the Krombein's hairy-footed mite (*Chaetodactylus osmiae*), which is transported to broods cells of *Osmia* by the female via phoresy (Seidelmann, 2006).

Whether or not these pests and diseases are contributing to population declines remains unclear, although a study by Cameron et al. (2011) observed that declining populations of North American bumble bees had significantly higher infection levels of the intracellular microsporidian pathogen *Nosema bombi* compared to non-declining species. Ingested spores of this introduced pathogen infect the gut and Malpighian tubules of infected workers, proliferating within the host. Fresh spores are released from decaying hosts or faeces and subsequently transmitted to other workers. Spread of this pathogen can result in the reduction of colony growth, fitness and reproduction.

1.3.3 Agricultural intensification

Large-scale changes in agricultural policies and practices such as increased grassland productivity and loss of hedgerows have caused the fragmentation and deterioration of many natural habitats relied upon by pollinators (Ollerton et al., 2014; Vanbergen, 2013). Particularly vulnerable are those species with more specialised diets and smaller foraging ranges such as solitary bees (Le Féon et al., 2013). Such agricultural intensification has been linked with pollinator extinction phases. For example, food security concerns during World War I prompted significant agricultural intensification, resulting in the greatest loss of bee species recorded (Ollerton et al., 2014; Williams and Osborne, 2009).

The use of insecticides to protect crops against herbivorous insects has played a predominant role in agricultural intensification, without which substantial yield losses would occur (Nauen and Bretschneider, 2002). There have been concerns that as insects, bees may be unintentionally harmed through various routes of exposure including plant (nectar, pollen, guttation water, and/or nesting materials) and non-plant (droplets of spray, particles in the air, water and/or soil) exposure routes (Brittain and Potts, 2011), although it must be

noted that the use of insecticides per se has been declining in the UK (see figure 4).

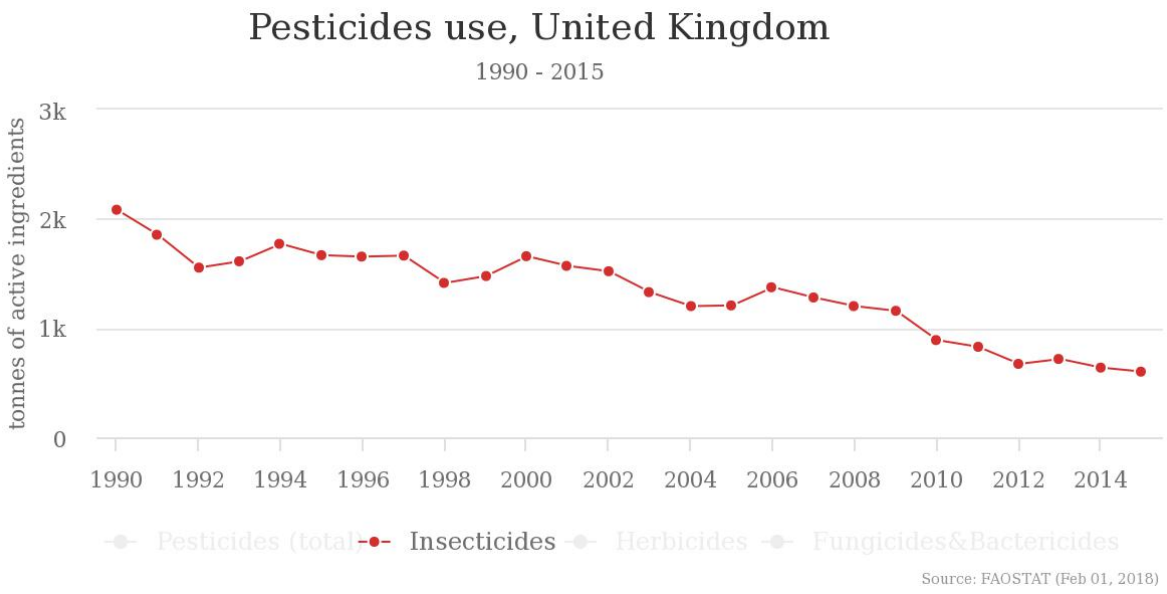


Figure 4. Tonnes of the active ingredient of insecticides used in the United Kingdom between 1990 and 2015. Taken from FAOSTAT (2018).

1.3.4 Summary

It is likely that the decline of pollinators is a result of an accumulation of the interacting factors outlined above and many others, which makes the explanation more difficult to establish. As such it is unwise to solely focus on one single factor as this could draw attention away from other issues (Mullin et al., 2010). Ecological models such as the BEEHAVE model developed by Rumkee et al. (2015) provide a useful tool to explore these interactions in a fully controlled stimulus. This particular model simulates how increased mortality of honey bees at various life stages due to insecticide use would impact colony dynamics.

Of the numerous factors implicated in bee declines, the impact of insecticides on bee health is probably the most strongly debated, and as such has generated an extensive amount of research (Cressey, 2017). A number of factors must be considered when assessing these data, including exposure

length (acute toxicity or sub-lethal affects), type of exposure (ingestion or topical) experimental conditions (field, semi-field, or laboratory) and species of bee. Moreover, each respective insecticide is unique, and should be assessed as such. The impacts of insecticides on bees are discussed in more detail in section 1.4.

1.4 Chemical control of insect pests

For centuries, herbivorous insects have been a major risk to food security and insecticides have played a predominant role in the protection of crops from these insects (Nauen and Bretschneider, 2002). Insecticides are chemicals that are able to kill, repel or adversely affect insects (Philogène, 1993). Early insecticides included inorganic sulphur (since 1000 BC) and arsenic (900 AD) (Casida and Quistad, 1998). In 1690, nicotine, in the form of tobacco leaf extracts, was recorded as the first plant-derived insecticide to be used for insect control. This was followed by pyrethrum in the 1800s, a natural insecticide made from the dried flowers of *Chrysanthemum cinerafolis* (Tomizawa and Casida, 2005). Synthetic insecticides have now largely taken their place and can be classified according to their mode of action (MoA). This classification system provides many organisations including government regulatory bodies and growers with information on what plant protection projects to use and when to use them (Sparks and Nauen, 2015). There are 29 known MoAs (IRAC, 2018a) acting on a variety of physiological aspects of insects such as the nervous system, respiration, growth and development, however only a few of these have any commercial value. Chemical classes with >10% market share include the neonicotinoids, pyrethroids and organophosphates (Nauen, 2006).

1.4.1 Insecticides targeting the nicotinic acetylcholine receptors

The nicotinic acetylcholine receptor (nAChR) is a nervous system receptor that interacts with the neurotransmitter acetylcholine (ACh). ACh is released from the presynaptic membrane and interacts with the binding site on the extracellular domain of the nAChR/ion channel complex. This causes conformational alterations in the receptor molecule leading to the opening of the

channel and the influx of Na^+ and the efflux of K^+. Acetylcholinesterases catalyse the breakdown of ACh, terminating the synaptic transmission (van der Sluijs et al., 2013).

1.4.1.1 Neonicotinoids

Neonicotinoids are a class of insecticides based on the structure of nicotine that act as agonists on the nAChR by mimicking ACh (van der Sluijs et al., 2013). Acetylcholinesterases are not able to catalyse the breakdown of neonicotinoids and thus overstimulation occurs, leading to paralysis and death of the insect (Casida and Durkin, 2013).

The neonicotinoid era began in the early 1990s with the development of the first commercially available compound, imidacloprid, by Bayer CropScience (Goulson, 2013). Since then modification of the prototype backbone has produced a number of other compounds including nitenpyram, acetamiprid, thiamethoxam, clothianidin and dinotefuran. In 2000 thiacloprid was added to the class, containing a cyanoiminothiazolidine skeleton instead of the original nitroiminothiazolidine skeleton (Kagabu et al., 2002). Neonicotinoids have become one of the most commercially important group of insecticides, accounting for over 25% of the world's insecticide market (van der Sluijs et al., 2013). Their success is due to a number of factors. Firstly, the greater specificity of insect nAChRs for neonicotinoids compared to those of vertebrates, resulting in selective toxicity to insects (Liu et al., 2010; Simon-Delso et al., 2015). Secondly, effective protection of the plant against insect pests only requires a small dosage; concentrations in plant tissues between five and ten ppb have been found to be effective at protecting plants against insect pests (Blacquière et al., 2012; Goulson, 2013). Finally, neonicotinoids are systemic. They are water soluble, and so are easily absorbed by plants either through their roots or leaves and are translocated throughout the plant, regardless of the mode of application. Subsequently, all components of the plant are protected from damage caused by a wide range of insect pests, such as direct damage from herbivores and also from indirect damage from plant viruses that may be transferred by insects (Elbert et al., 2008; Simon-Delso et al., 2015).

However, the systemic nature of these insecticides can also result in the inadvertent exposure of bee pollinators via consumption of pollen and nectar containing trace levels of insecticide, with average trace levels of 2 µg/kg of neonicotinoids in nectar, and 3 µg/kg in pollen (Blacquière et al., 2012). Additionally, pollinators can be exposed through environmental contamination, which can occur through a number of routes such as dust emission during seed drilling (Nuyttens et al., 2013), guttation water (Girolami et al., 2009), contamination of wild plants near crops (Botías et al., 2016), and also presence in the soil. Studies quantifying the uptake of neonicotinoids from seed dressings into the plant indicate that the majority of active compound enters the soil, with only 1.6-20% absorbed by the plant (Goulson, 2013). Neonicotinoids can remain present in the soil for some time, ranging from 100 days to 3.4 years (imidacloprid) depending on factors such as soil type, temperature and pH (Bonmatin et al., 2015). Female *O. bicornis* frequently use mud to construct partitions of brood cells within a nest (Seidelmann, 2014), and thus the larvae could be exposed to accumulating levels of neonicotinoids from soils.

Neonicotinoids can be further classified according to their pharmacophore system into three chemical groups; the *N*-nitroguanidines (imidacloprid, thiamethoxam, clothianidin and dinotefuran), nitromethylenes (nitenpyram), and *N*-cyanoamidines (acetamiprid and thiacloprid) (Goulson, 2013). Studies have found that bee pollinators can exhibit profound differences in their sensitivity to these compounds, with the *N*-nitroguanidines found to be more toxic than *N*-cyanoamidines to honey bees when topically applied (Iwasa et al., 2004). During this study imidacloprid, thiacloprid and acetamiprid were used as examplars of nitro- and cyano-substituted compounds.

Imidacloprid is the most well-studied neonicotinoid compound, with the majority of studies concluding that imidacloprid is highly toxic to honey bees when topically applied (Bailey et al., 2005; Biddinger et al., 2013; Decourtye and Devillers, 2010; Iwasa et al., 2004). Although, a meta-analysis of 14 studies by Cresswell (2011) concluded that imidacloprid does not seem to confer lethality at field realistic doses (1-10 ppb), but does seem to have sublethal effects. Exposure of honey bees to sublethal levels of imidacloprid has been found to affect sperm viability (Chaimanee et al., 2016), learning performance (Decourtye et al., 2003), and flight (Girolami et al., 2012). Fewer studies have

been carried out examining the effects of thiacloprid and acetamiprid, but they have generally been found to show a low acute toxicity to honey bees (Badawy et al., 2015; Iwasa et al., 2004; Laurino et al., 2011).

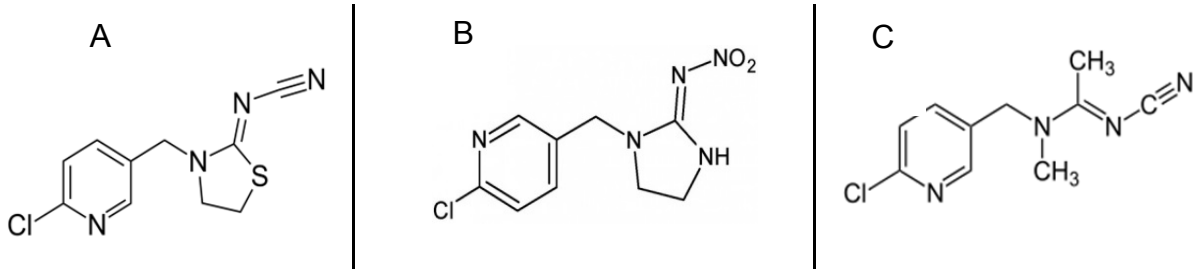


Figure 5. Chemical structures of the neonicotinoid insecticides used in this study; thiacloprid (A), imidacloprid (B) and acetamiprid (C).

1.4.1.2 Butenolides

Butenolides are a class of insecticides based on the natural product stemofoline, which is isolated from the stem and leaves of *Stemona japonica*. Launched in 2014, flupyradifurone (Sivanto® prime) is the first representative of the butenolide class. Although this class has the same mode of action as neonicotinoids, it differs in its pharmacophore system and structure-activity-relationships (SAR) and thus is classified as a new chemical sub-group 4D (Nauen et al., 2015; IRAC, 2018a).

Flupyradifurone has been found to be practically non-toxic to bumblebees and honey bees when topically applied, however bees appear to be more susceptible when exposed orally (Nauen et al., 2015).

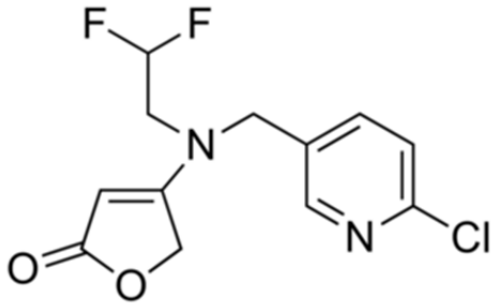


Figure 6. Chemical structure of flupyradifurone.

1.4.2 Insecticides targeting voltage-gated sodium channels

Voltage-gated sodium channels (VGSC) are integral membrane proteins that form ion channels. In response to an action potential, the channel undergoes three conformational states; closed, open and deactivated. During normal resting potential the ion channels are in their deactivated state, with their activation gates closed. When an action potential occurs the activation gate opens, allowing the influx of Na^+, and the depolarisation of the neuron. When a sufficient amount of Na^+ has entered the neuron, the channels inactivate themselves by closing the inactivation gates. The potential then decreases back to its resting potential and is repolarised with the activation gate closed and the inactivation gate reopened (Davies et al., 2007). The VGSC is the target-site for a number of insecticide classes such as DDT, pyrethrins and pyrethroids.

1.4.2.1 Pyrethroids

Pyrethroids act on the alpha-subunit of the voltage-gated sodium channel, causing prolonged opening of the channel and the continuous conduction of Na^+. Such repetitive simulation of the channel leads to paralysis and death of the insect.

Pyrethroids are derived from the six insecticidal esters of chrysanthemic and pyrethric acid found in *Chrysanthemum cinerafolis*, a herbaceous perennial of the family Compositae (Casida, 1980). Unlike nicotine which is found in the

pollen and nectar of plants, pyrethrins are isolated in the secretory ducts of the achenes, preventing any detrimental effects to pollinators (Casida, 1980). The dried flowers of this species are thought to have been used to control insect pests in ancient China as far back as the 1st century (Davies et al., 2007), however their large-scale use was limited due to their low stability in light and air. The first synthetic pyrethroid, permethrin, was developed at Rothamsted Research in 1973, shortly followed by cypermethrin and deltamethrin (Elliott et al., 1978).

Pyrethroids are broad-spectrum insecticides, controlling a wide variety of insect pests, but can persist in the environment for quite some time due to their resistance to photodecomposition (Casida, 1980; Elliott et al., 1978). In this study, two members of the pyrethroid class, deltamethrin and tau-fluvalinate, were used.

Due to its relatively low acute toxicity to bees, tau-fluvalinate is commonly used in-hive to treat infestations of the bee parasite *Varroa destructor* (Johnson et al., 2006). However, sublethal doses of tau-fluvalinate (LD_{10}) have been found to cause locomotor deficits in day-old honey bees (Charreton et al., 2015), challenging its use. Bees have been found to be more susceptible to deltamethrin compared to tau-fluvalinate, although data are scarce (Decourtye et al., 2004; van dame et al., 1995).

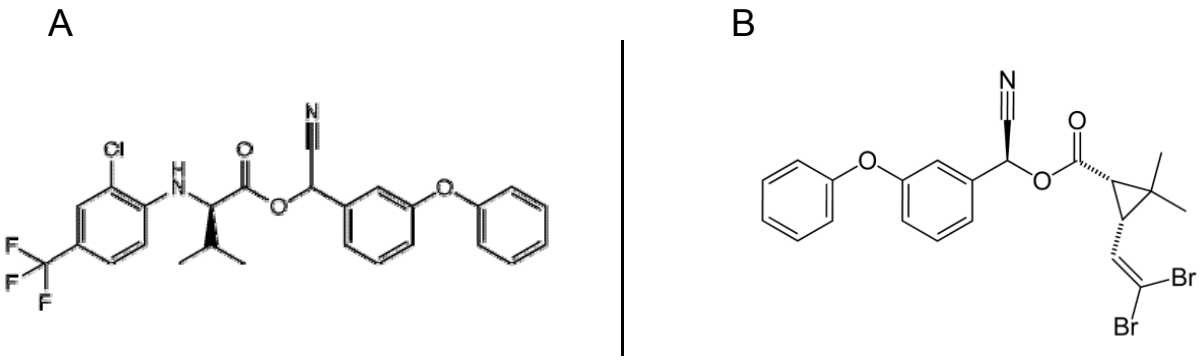


Figure 7. Chemical structures of pyrethroids used in this study; tau-fluvalinate (A) and deltamethrin (B).

1.4.3 Insecticides targeting acetylcholinesterases

Acetylcholinesterases are enzymes that catalyse the hydrolysis of the neurotransmitter ACh into choline and acetic acid. Following a nerve impulse acetylcholine is released into the synaptic junction from its vesicle. Within 2-3 milliseconds acetylcholinesterase interacts with the ACh receptor site and synaptic transmission is disrupted (Fukuto, 1990).

1.4.3.1 Organophosphates

Organophosphates act by inhibiting acetylcholinesterase. When the enzyme is inhibited, the concentration of ACh remains high, resulting in the continuous stimulation of the muscle/nerve fiber and eventually death of the insect (Fukuto, 1990). These insecticides are esters of phosphoric acid. The first organophosphate, tetraethylpyrophosphate (TEPP), was produced in 1854, and although highly toxic, it is also hydrolysed rapidly (Casida and Quistad, 1998). Since then many other organophosphorus compounds have been developed. During this study the organophosphates coumaphos and chlorpyrifos were used. Like tau-fluvalinate, coumaphos is of such low acute toxicity to honey bees that it is commonly used to treat *Varroa* infestations (Calatayud-Vernich et al., 2016). However, long-term use can cause coumaphos to build up in the wax of colonies, which has been linked to impairment of olfactory learning and memory in honey bees (Williamson and Wright, 2013). Chlorpyrifos on the other hand, has been found to be substantially more toxic to bees than coumaphos (Schmehl et al., 2014).

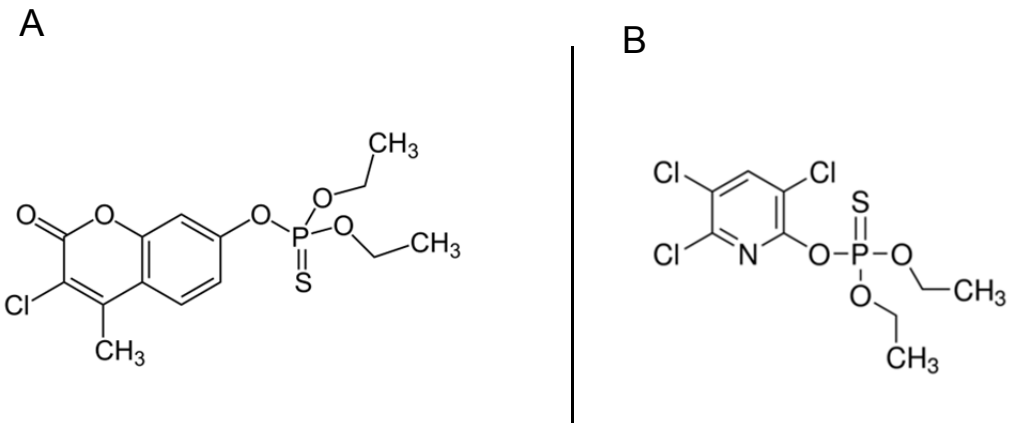


Figure 8. Chemical structures of the organophosphates coumaphos (A) and chlorpyrifos (B).

1.4.4 Insecticide synergists

An insecticide synergist is a substance that, whilst not being toxic itself, can be used to enhance the toxicity of an insecticide. Synergists are often used in chemical mixtures applied to crops to either decrease the effective dose of insecticide applied, or to reinstate the lethality of the insecticide when a population has developed resistance mediated by metabolic mechanisms. During this study the synergist Piperonyl butoxide (PBO) was used to initially explore the possible detoxification mechanisms of *O. bicornis* by P450 enzymes. PBO is a synthetic methylenedioxyphenyl compound derived from benzodioxole. Introduced in 1947, PBO was the first commercially available synergist, initially produced in an attempt to stabilise pyrethrins (Casida, 1970; Philogène, 1993). PBO acts by preventing the binding of insecticides to cytochrome P450 enzymes (see section 1.5.3.1 for more details on P450s), and inhibiting their detoxification.

1.4.5 Alkaloids

Alkaloids are a group of naturally occurring low molecular weight compounds that contain nitrogen (Stevenson et al., 2017). These secondary compounds are produced by plants and largely serve as a chemical defence against herbivorous insects (Johnson et al., 2010). They are predominantly found in the leaves, anthers and flowers of plants, but are also found at lower concentrations

in the pollen and nectar that bees collect (Cook et al., 2013). For example, nicotine is a plant secondary metabolite produced by *Nicotiana tabacum* (tobacco) (du Rand et al., 2015). Leaves of this species have been found to contain up to 2730 µg/g of nicotine, whereas the pollen and nectar of this species contains only 22.7 µg/g and 0.2 µg/g respectively (Detzel and Wink, 1993). As the LD_{50} (dose required to cause 50% mortality of test subjects) of this alkaloid is 2,000 ppm against honey bees (Detzel and Wink, 1993), exposure to naturally occurring concentrations of nicotine is unlikely to cause harm to bees (as demonstrated by Singaravelan et al. (2006)). During this study the ability of *O. bicornis* to break down the alkaloids nicotine, anabasine, atropine, and hyoscine was assessed.

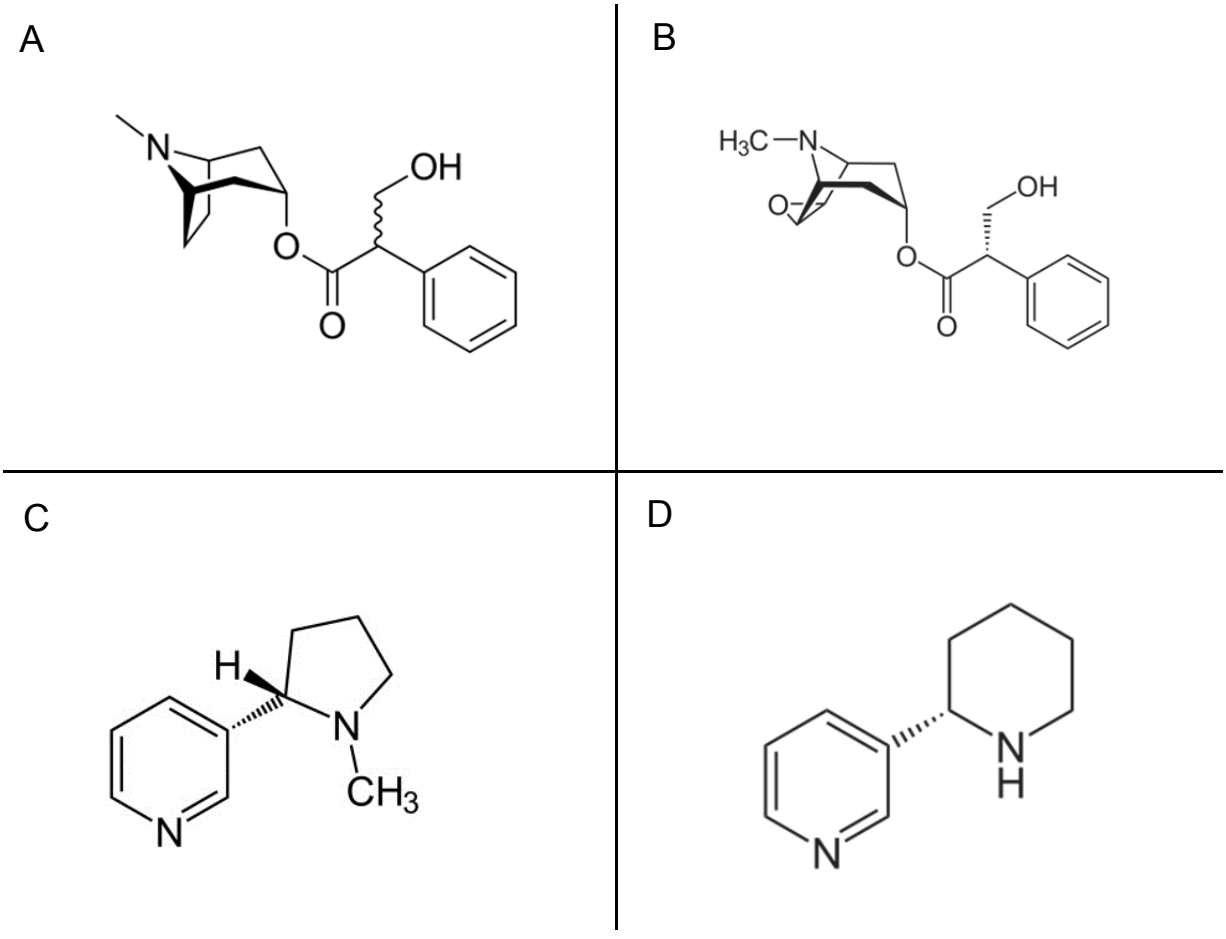


Figure 9. Chemical structures of the alkaloids nicotine (A), anabasine (B), atropine (C) and hyoscine (D).

1.5 Insecticide resistance mechanisms

Insecticide resistance can be defined as “a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species” (IRAC, 2018b). Insecticide resistance has been an agrochemical issue for more than half a century, with the first study documenting insecticide resistance published over 100 years ago. Since then the number of resistance cases has continued to rise, and the research on this topic has become substantial (Melander, 1914; Sparks and Nauen, 2015). This has resulted in the depiction of four main heritable resistance mechanisms (described below), and provides a valuable starting platform from which the molecular basis of bee sensitivity to insecticides can be explored.

1.5.1 Target-site resistance

Target-site resistance refers to a scenario in which amino acid changes at the target-site result in an alteration of its structure, causing ineffective binding of the insecticide to its target (Pittendrigh et al., 2008). This is a fairly common mechanism of resistance, and has been reported in 12 target-sites encompassing 11 of the 29 MoA outlined by IRAC (2018a). The majority of documented cases involve point mutations, and most are for insecticides targeting the voltage-gated sodium channel (IRAC, 2016).

1.5.2 Penetration resistance

Penetration resistance occurs when an insect develops a heritable mechanism that reduces the penetration of an insecticide through the cuticle and into the insect’s body. This is mainly achieved by cuticular thickening or modification of the cuticle composition, e.g. by an increased deposition of cuticular hydrocarbons (CHCs) on the epicuticle, which is thought to allow detoxification

enzymes more time to process toxins (Balabanidou et al., 2018; Bass and Jones, 2016).

Only a small number of cases of insecticide resistance have been attributed to changes in penetration ability, such as neonicotinoid resistance of the peach-potato aphid *Myzus persicae* (Puinean et al., 2010), and deltamethrin resistance of the cotton bollworm *Helicoverpa armigera* (Ahmad et al., 2006) (see recent review by Balabanidou et al. (2018) for other examples). These cases are often reported in combination with other resistance mechanisms. One noteworthy study by Balabanidou et al. (2016) assessed the role of cuticle changes in the resistance of *Anopheles gambiae* to deltamethrin. They found that the penetration of [^{14}C]detamethrin was ~50% lower in resistant compared to susceptible strains. Importantly, they provided evidence for the role of P450s (CYP4G16 and CYP4G17) in CHC synthesis, which were found to be highly abundant in oenocytes, a cell type thought to secrete hydrocarbons.

1.5.3 Metabolic resistance

The majority of insecticide resistance cases are metabolic in nature, occurring due to an increase in the number or efficiency of the enzymes catalysing the breakdown of insecticides into less toxic metabolites. Xenobiotic detoxification can be divided into two general phases: phase I metabolism, which involves the initial modification of a xenobiotic, commonly involving hydroxylation reactions by cytochromes P450 (P450s) and carboxylesterases (CCEs), and phase II metabolism, which refers to conjugation reactions often catalysed by glutathione-S-transferases (GSTs) (Panini et al., 2016). The functions of these enzymes are outlined below.

1.5.3.1 Carboxyl/Cholinesterases

Carboxylesterases and cholinesterases are esterases that catalyse the hydrolysis of carboxylic and choline-based esters respectively. Mutated esterases have commonly been implicated in organophosphate and carbamate insecticide resistance. Evidence suggests that this resistance arises either through changes in the CCE amino acid sequences or by amplification of CCE genes (Tsubota and Shiotsuki, 2010). The detoxification group of CCEs includes acetylchloinesterases which were mentioned previously. These are esterases that catalyse the breakdown of neurotransmitter acetylcholine (Mutero et al., 1994).

1.5.3.2 Glutathione-S-transferases

Glutathione-S-transferases (GSTs) are a major group of enzymes found in almost all living organisms. GSTs are able to catalyse the conjugation of electrophilic compounds with the thiol group of reduced glutathione, increasing their solubility and thus excretion ability (Han et al., 2016). In this way, these enzymes are often involved in the detoxification of xenobiotics. Elevated expression of GSTs has been associated with insecticide resistance in all major insecticide classes (Kostaropoulos et al., 2001; Yang et al., 2016; Zhu et al., 2015).

1.5.3.3 Cytochrome P450 enzymes

Cytochromes P450, or CYP genes, comprise one of the largest families of genes and are present in the genomes of virtually all living organisms. The number of P450 genes represented within an organism's genome (known as the CYPome) is highly variable (Berenbaum, 2002; Werck-Reichhart and Feyereisen, 2000). For example, humans have a CYPome of 57 functional CYP genes, whereas mice have a CYPome of 102 functional CYP genes (Nelson et al., 2004). The first insect genome to be sequenced was that of the fruit fly (*Drosophila melanogaster*), which was found to contain 90 P450 genes, seven of which are thought to be pseudogenes (Good et al., 2014; Tijet et al., 2001a).

The P450 enzymes are a large superfamily of heme-thiolate proteins that are able to metabolise a wide range of both endogenous and exogenous hydrophobic compounds, and thus are extremely versatile (Berenbaum, 2002). They are commonly described as “environmental response genes” (as defined by Berenbaum (2002)). Features of environmental response genes include (1) very high diversity, (2) proliferation by duplication events, (3) high rates of evolution, and (4) tissue- or temporal-specific expression (Berenbaum, 2002; Feyereisen, 2012). They are best known for being monooxygenases, catalysing the transfer of an atom of oxygen to a substrate and reducing the other to water. Other roles of these enzymes include oxidases, reductases, and isomerases (Feyereisen, 2012).

P450 genes are named after their common feature: an absorbance peak of their Fe^{II}-CO complex, around 450 nm (Feyereisen, 2012). The nomenclature system, which was introduced by Nebert (1987), states that all members of a family share 40% identity, whereas those members of a subfamily share 55% identity, and those with over 97% identity are identified as allelic variants at a locus (Berenbaum, 2002; Tijet et al., 2001a; Figure 10). Analysis of available sequences suggests that insect CYP genes typically fall into four main clades or “clans”; the CYP2 clade, CYP3clade, CYP4 clade and the mitochondrial clade (Feyereisen, 2012; Figure 11). The highly diverse CYP3 clade, which includes the large CYP6 and CYP9 families, are most commonly involved in insecticide metabolism and resistance. The proximity of these gene sequences on chromosomes 13 and 14 in *A. mellifera* along with the number of conserved introns suggests that they are the result of fairly recent gene duplication events (Claudianos et al., 2006).

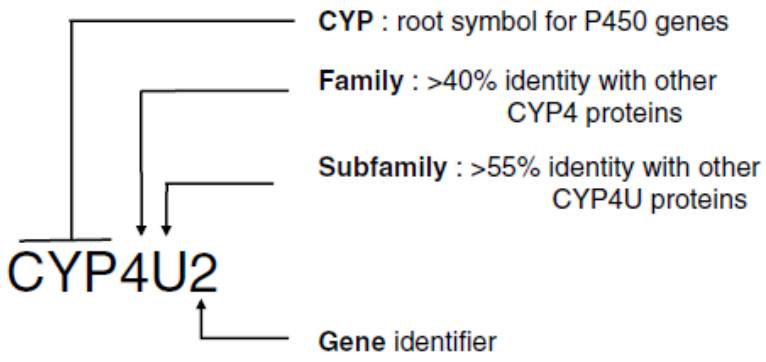


Figure 10. Diagram of the Cytochrome P450 nomenclature system. Taken from Feyereisen (2006).

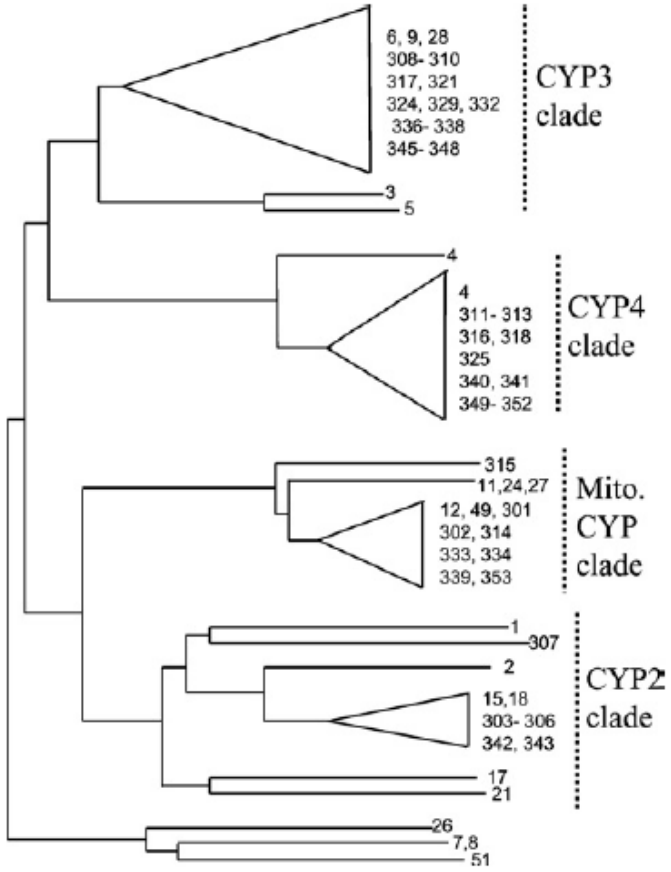


Figure 11. Diagram of the four clades of insect Cytochromes P450. Taken from Feyereisen (2006).

1.5.4 Behavioural resistance

In some cases, insects have been found to avoid ingesting toxic compounds altogether by detecting them visually, olfactorily or via contact (Després et al., 2007). This mechanism is more difficult to validate; to meet the definition of insecticide resistance devised by IRAC studies must demonstrate a “heritable change in the sensitivity of a pest population” (IRAC, 2018b) and as such clearly validated cases of behavioural resistance to insecticides are scarce (Zalucki and Furlong, 2017). Most studies seem to demonstrate aversion behaviours, but assume rather than prove the genetic basis of the trait (e.g. (Fornadel et al., 2010; Sarfraz et al., 2005; Stapel et al., 1998).

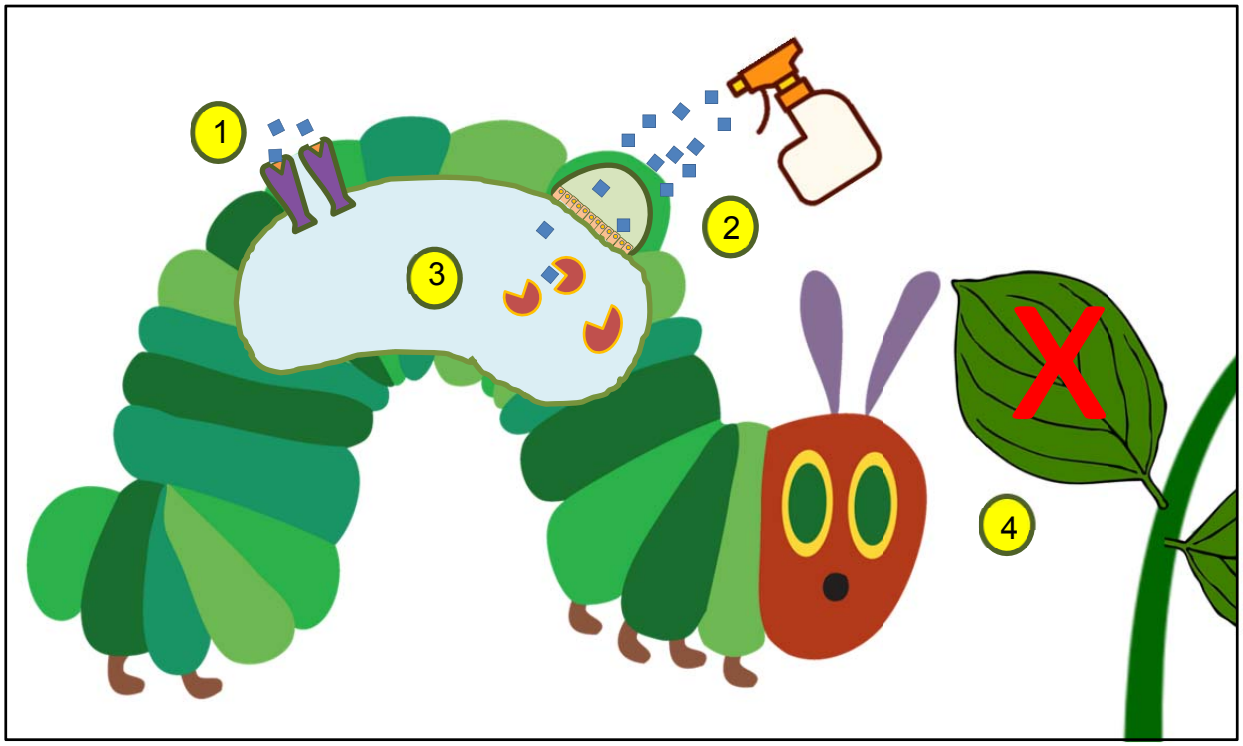


Figure 12. Schematic illustration of the mechanisms through which insecticide resistance can develop: 1) Target-site resistance, 2) penetration resistance, 3) metabolic resistance, and 4) behavioural resistance.

1.6 Objectives of the project

The overall aim of this project was to characterize the molecular and biochemical basis of the differences in the susceptibility of the solitary bee *O. bicornis* to select insecticides. Specifically, this thesis aimed to:

1. Evaluate the susceptibility of *O. bicornis* to select insecticides through topical bioassays and the possible role of P450 enzymes in observed differences in sensitivity between compounds using synergist bioassays.

2. Explore the pharmacokinetics of select insecticides by a) exploring their cuticular penetration ability *in vivo* and b) determining their binding affinity to the nicotinic acetylcholine receptor *in vitro*.

3. Assess the capacity of *O. bicornis* to detoxify select insecticides and alkaloids by a) using whole microsomes and b) by identifying candidate P450 detoxification genes in the transcriptome and genome of this species.

4. Functionally characterise select detoxification genes *in vitro* by expression in an insect cell line, and *in vivo* by the creation of transgenic *Drosophila* containing candidate genes.

5. Further investigate the detoxification of natural and synthetic insecticides by *O. bicornis* by a) quantifying the tissue-specific expression of candidate genes, and b) exploring the induced upregulation of genes after application of neonicotinoids.

Chapter two: General methods

2.1 Centrifugation

All small volume centrifugation steps were carried out in a 5415D model centrifuge, or a 5415R model centrifuge (Eppendorf) when temperature control was needed. Centrifugation of volumes larger than 2 mL were carried out in a 5810R table top centrifuge (Eppendorf).

2.2 RNA extraction

Approximately 30 mg of flash frozen adult *O. bicornis* females (Dr. Schubert Plant Breeding, Landsberg, Germany) were ground in a 1.5 mL Eppendorf tube placed on dry ice using a pre-cooled sterile polypropylene pellet pestle. RNA was extracted using an RNeasy Plus kit (Qiagen) or a Bioline Isolate II RNA Mini Kit (Bioline Reagents) following the manufacturer's recommended protocols. The RNA was resuspended in either 50 µL of Tris-EDTA buffer or nuclease-free water, depending on subsequent use.

The quality of RNA was assessed by A260/280 and A260/230 OD measurements on a NanoDrop1000 spectrophotometer (ThermoFisher scientific) or a NanoDrop One (ThermoFisher scientific). Samples were accepted as pure when the A260/230 ratios were around 1.8. The integrity of RNA was analysed by visualisation on a 1% TAE buffer agarose gel. 1 µL of RNA samples were incubated with 1µL of 6X RNA loading dye (New England Biolabs) and 4 µL of RNase-free water at 65°C for 5 minutes. Samples were run on a 1% TAE agarose gel containing 2.5 µL of RedSafe stain (x20,000) (Chembio) at 80V/200 mA for 40 minutes. All RNA samples were stored at -80°C.

2.3 First-strand cDNA synthesis

SuperScript® III Reverse Transcriptase (Invitrogen) or Maxima H Minus First Strand cDNA Synthesis kit (ThermoFisher scientific) was used to synthesise

template cDNA for use in PCR and qPCR reactions according to the manufacturer's protocol. 20 µL reactions were carried out in 0.2 mL thin-walled PCR tubes (4titude) containing the following components: 4 µg of total RNA, 1 µL of random primers, 1 µL of dNTP mix (10 mM) and nuclease-free water (up to 13 µL). cDNA samples were stored at either -20°C (Invitrogen) or -80°C (ThermoFisher scientific).

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Primer design

All gene-specific primers were designed using Geneious (Biomatters Limited), adhering to the following guidelines where possible. Primers should be around 18-22 base pairs (bp) in length, the Primer Melting Temperature (T_m) should be in the range of 52-58°C, and primers used in the same reaction should have similar T_m values. The GC content of primers should be 40-60% and if possible contain a GC clamp at the 3' end. Primers were also checked for secondary structures using the online tool OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html). All primers were synthesised by Sigma-Aldrich (Haverhill facility, UK) and supplied at a concentration of 100 µM in water. Working concentrations of primers were 10 µM diluted in nuclease-free water (see appendix 2 for primer sequences). All primers were stored at -20°C.

2.4.2 Polymerase chain reaction (PCR)

All PCR reactions were carried out in 0.2 mL thin-walled PCR tubes containing 1 µL (5-10 ng) of cDNA template, 1 µL of forward and reverse primers (1 pmol), 12.5 µL of DreamTaq PCR Master Mix (ThermoFisher Scientific), and nuclease-free water to a total volume of 25 µL. Other enzymes used for amplification of fragments included *Pfu* DNA polymerase (Promega) and Long-Range PCR mix (Thermo-Fermentas) depending on application. All PCR reactions were run alongside a control containing nuclease-free water instead of the cDNA template.

All PCR reactions were set up on ice and carried out using a thermal cycler with a heated lid (G-Storm GS1 Thermal cycler, ThermoFisher, or T100™ Thermal Cycler, Bio-Rad). General PCR reaction temperature cycling conditions were 95°C for 3 minutes (initial denaturation), followed by 35 cycles of 95°C for 30 seconds (denaturation), 60°C for 20 seconds (annealing), and 72°C for 30 seconds (elongation), with a final elongation step at 72°C for 5 minutes.

2.4.3 Quantitative Real-Time Polymerase Chain Reaction

Quantitative polymerase chain reactions (qPCR) was carried out using a Rotor-Gene 6000™ (Corbett Research) or a CFX Connect™ Real-time PCR system (Bio-rad) with the following thermocycling conditions: 3 minutes at 95°C followed by 40 cycles of 95°C for 20 seconds (denaturation), 60°C for 20 seconds (annealing), and 72°C for 30 seconds (elongation). A final melt-curve step was included to rule out any non-specific amplification. All qPCR reactions were set up on ice and consisted of 6 µL cDNA (10 ng), 7 µL of 2X SYBR™ Green PCR Master Mix (ThermoFisher Scientific), 0.25 µM of forward and reverse primers and nuclease-free water to a total volume of 15 µL. Primers were designed to amplify a ~200 bp section of each target gene with low percentage identity to other target genes. All primers were designed using the Primer3 primer design tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and are listed in appendix 2. Prior to qPCR experiments, the efficiency of each primer pair was assessed using a series of dilutions of cDNA (0.01-100 ng) to generate a standard curve. Each qPCR experiment consisted of four biological replicates and two technical replicates. Controls consisted of reactions containing nuclease-free water in the place of cDNA template.

2.5 Restriction digests

Restriction digests were carried out in 0.2 mL thin-walled PCR tubes according to the manufacturer's protocol. When carrying out double digests compatibility of the two enzymes and their buffers were checked using the manufacturer's information.

2.6 Agarose gel electrophoresis

PCR products, digestion products, and baculovirus recombination products were examined by electrophoresis. Gels were made up from genetic analysis grade agarose (Fisher Scientific) and Tris Acetate EDTA (TAE) buffer (ThermoFisher Scientific) at 1% w/v, heated in a microwave to boiling point, stirred and left to cool. All cast gels were stained with either 10 mg/mL ethidium bromide or 2.5 µL (x20,000) RedSafe and transferred to a casting system to set. When products did not contain loading dye 5 µL of the sample was mixed with 1 µL of 6X Gel Loading Dye (NEB). All products were visualised on a 302 nm UV trans-illuminator (Syngene or Bio-Rad). A 1 kb Plus DNA ladder (ThermoFisher scientific) was run in a single lane alongside samples in order to estimate the molecular mass of the samples. PCR and baculovirus recombination products were run at 80V/200 mA for 40 minutes, and digestion products were run at 60V/200 mA for 1 hour or more.

2.7 Cloning

2.7.1 Ligation

DNA fragments were ligated into vectors in 0.2 mL thin-walled PCR tubes containing 1 µL of T4 DNA ligase (5U/µL) (NEB), 2 µL of 10X T4 DNA Ligase Buffer (NEB), a 3:1 insert:vector molar ratio calculated using NEBiocalculator (https://nebiocalculator.neb.com/#!/ligation), and nuclease-free water to a total volume of 20 µL. Reactions were incubated at 16°C overnight and heat inactivated at 65°C for 10 minutes.

2.7.2 Transformation

10 ng of ligation reactions (diluted in 10 mM Tris-HCL, pH 7.5) were transformed using 100 µL of Library Efficiency™ DH5α™ Competent Cells (Invitrogen) or MAX Efficiency™ DH5α™ Competent cells (Invitrogen) (depending on the size of the plasmid) following the manufacturer's protocol. 100 µL and 40 µL of the transformation mixture were spread onto LB plates (see appendix 1) containing the appropriate antibiotic and incubated at 37°C

overnight.

2.7.3 Colony PCR

PCR was used to determine the presence of insert DNA in transformed bacterial plasmid constructs using primers designed to specifically target the insert DNA. These reactions used DreamTaq PCR Master Mix (ThermoFisher Scientific) as described in 2.4.2 but with bacterial cells used as a template. A sterile p10 pipette tip was used to transfer individual bacterial colonies from LB agar plates to a fresh LB agar plate containing the appropriate antibiotics and then into a PCR mastermix. Plated colonies were incubated at 37°C overnight. Typical PCR cycling conditions were as described in 2.4.2. 5 µL of PCR product was run on a 1% TAE agarose gel to confirm the presence of the DNA insert. 12 colonies were typically screened for each insert.

2.8 PCR purification

2.8.1 Gel extraction

PCR products were run on a 1% (TAE) agarose gel (see above) for 40-60 minutes. The desired bands were excised from the agarose gel using a scalpel under a blue light safe imager (Clare Chemical Research) and placed in a 1.5 mL Eppendorf tube. Extraction and purification of DNA was carried using the QIAquick Gel Extraction kit (Qiagen) or the DNA Gel Extraction Kit (Monarch) following the manufacturer's recommended protocols. The quantity and quality of products were assessed using a NanoDrop1000 spectrophotometer (ThermoFisher Scientific) or a NanoDrop One (ThermoFisher Scientific).

2.8.2 Column purification

Single *E. coli* colonies were selected from freshly streaked plates from colony PCR and transferred to liquid cultures grown in 15 mL Falcon tubes containing 5 mL of low-salt LB broth (see appendix 1) supplemented with 50 µg/mL of the relevant selection antibiotic and incubated at 37°C, 100 rpm for a minimum of 16 hours. Cells were then pelleted by centrifugation at 3,500g for 15 minutes at

15°C. A GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific) was used to purify samples following the manufacturer's protocol. The quantity and quality of products were assessed using a NanoDrop1000 spectrophotometer (ThermoFisher Scientific) or a NanoDrop One (ThermoFisher Scientific).

2.9 Sequencing

Sequencing was carried out by Eurofins Genomics using their TubeSeq service. Premixed samples (DNA and primer) were sent according to their sample submissions guide.

2.10 Bradford protein assay

The concentrations of protein extracted from microsomal preparations were determined according to Bradford (1976) using Bovine Serum Albumin (BSA) as the protein standard (Sigma-Aldrich). Bradford protein assays were carried out in clear, flat-bottomed 96-well plates (CytoOne). A 25 µL serial dilution of protein standard and microsomal protein was carried out using potassium buffer (1, 0.5, 0.25, 0.125, 0.0625 mg/mL and 1 in 1, 1 in 2, 1 in 4, 1 in 8, 1 in 16 respectively). Three replicates were carried out for each sample. 5 µL of each dilution was transferred to a new well and mixed with 200 µL of Bradford reagent (Sigma). Samples were left at room temperature for 15-20 minutes. The absorbance readings were measured using a SpectraMax Gemini XPS Microplate Reader (Molecular Devices) at 595 nm.

Chapter three: Sensitivity of *O. bicornis* to select insecticides

Statement of contribution

The data detailed in this chapter was collected with the assistance of Emma Randall (year 1), Laura Kor (year 2) and Becky Reid (year 3).

3.1 Introduction

Monitoring the sensitivity of insects to insecticides is an important part of both insecticide registration and resistance monitoring. This is frequently achieved through bioassays, which involve exposing insects to a range of doses of an insecticide and recording the observed mortality over a certain period of time. These data are used to generate a dose-response curve from which the lethal dose of insecticide that results in a 50% mortality of test subjects (LD_{50}) can be calculated. The Organisation for Economic Co-operation and Development (OECD) has developed such methodologies for assessing the toxicity of insecticides to adult worker honey bees. The OECD guidelines describe two toxicity tests differing in their mode of insecticide application. These tests intend to reflect two of the possible exposure routes of bees to insecticides; contact exposure (e.g. contaminated nesting materials/droplets of spray) and oral exposure (e.g. through consumption of contaminated pollen and nectar) (Brittain and Potts, 2011). The U.S. Environmental Protection Agency (EPA) has also developed a classification system to assess the toxicity of an insecticide based on its LD_{50} values, ranging from 'practically non-toxic' (>100 µg/bee) to 'highly toxic' (<2 µg/bee) (see table 1). This chapter describes the adaptation of the OECD protocols to enable the assessment of the acute contact toxicity of adult *O. bicornis* females and extrapolation of the terminology developed by the EPA to assess the LD_{50} values generated.

Very few insecticide toxicity tests have been carried out on solitary bee species (e.g. Arena and Sgolastra (2014); Gradish et al. (2012); Hodgson et al. (2011)) and even fewer on *Osmia* spp. (e.g. Biddinger et al. (2013); Scott-Dupree et al. (2009)). To date, only one contact study has been carried out on *O. bicornis* adults. This study investigated the impact of sublethal exposure to thiamethoxam and clothianidin (concentrations of 2.87 µg/kg and 0.45 µg/kg respectively) on lifetime reproductive success. A 50% decrease in total offspring

production was observed along with an increase in the number of male offspring produced (Sandrock et al., 2014).

As mentioned in section 1.3.1.1, studies have found that bee pollinators can exhibit profound variation in their sensitivity to different insecticides within the same class. In the case of the neonicotinoids, *N*-nitro-guanidines (e.g. imidacloprid LD_{50}= 0.018 µg/bee) have been found to be orders of magnitude more toxic than *N*-cyano-amidines (e.g. thiacloprid LD_{50} = 14.6 µg/bee) to honey bees when topically applied (Iwasa et al., 2004). This differential bee sensitivity has been shown to be determined by cytochrome P450 monooxygenases (P450s), specifically by a single P450, CYP9Q3, identified as a highly efficient metaboliser of thiacloprid (Manjon et al., 2018). As mentioned in chapter 1.4.4, PBO is an insecticide synergist known to inhibit P450 enzyme activity, and thus has become a valuable tool for investigating the involvement of P450s in insecticide detoxification (Casida, 1970). The aim of this chapter was to 1) determine whether this phenomenon extends to the solitary bee pollinator *O. bicornis*, and to 2) explore the possible role of P450 enzymes in this differential bee sensitivity by carrying out topical synergist bioassays with PBO.

LD_{50} value	**Classification**
<2 µg/bee	Highly toxic
>2 - <11 µg/bee	Moderately toxic
11 – 100 µg/bee	Slightly toxic
>100 µg/bee	Practically non-toxic

Table 1. Terminology guidelines for insecticide toxicity to bee pollinators based on LD_{50} values (EPA, 2014).

3.2 Methods

3.2.1 Bee care and maintenance

For three consecutive years of this PhD 7,000 ± 500 *O. bicornis* cocoons were purchased from Dr. Schubert Plant Breeding (Landsberg, Germany). These were field-collected pupae that were obtained by placing 'trap' nests in the wild. Upon arrival, loose cocoons were stored in aerated plastic containers (12 cm X 8 cm) containing groups of ~ 200 cocoons at 4°C in complete darkness. Cocoon size ranged from 0.5-1.7 cm in length. During the first year, emergence of *O. bicornis* was initiated on 1st April, however this was found to be too early as high levels of mortality were observed. The optimal time to begin emerging bees was found to be 1st May and lasted through to 1st July. The bees were emerged in staggered batches of 100-200 bees at a time. Cocoons were individually transferred into aerated clear plastic cages (10cm X 7cm) with ~ten cocoons in each cage. The cages were placed in an incubator (25°C; 50% relative humidity; L16:D8) to allow bees to emerge. Upon hatching, individuals were sexed according to the colour of their clypeus (females: black, males: white; Seidelmann (2014)). Males were discarded and females were fed 50% sucrose solution soaked into cotton wool in a blue plastic dish (see figure 13).

3.2.2 Topical insecticide bioassays

Technical grade thiacloprid, imidacloprid and flupyradifurone were obtained from Bayer CropScience (Germany) and technical grade acetamiprid, tau-fluvalinate, deltamethrin, coumaphos and chlorpyrifos were purchased from Sigma. The selection of these compounds for testing was based on published toxicity data of other bee species.10 µg stock solutions were made by diluting insecticides in acetone. The stock solutions were stored in the dark at 4°C in a refrigerator for no longer than 3 weeks prior to use. Pyrethroids are known to bind strongly to materials like plastic and thus stocks of these compounds were made fresh prior to each bioassay. The stock solutions were used to produce the concentration ranges shown in table 2. The following method was adapted from the OECD test guidelines for honeybees (1998). Prior to commencing

bioassays, *O. bicornis* adult females of roughly the same age (~24 hours old) underwent a 24-hour adaption period to housing conditions. They were then anaesthetised with CO_2 for 5-10 seconds to allow application of insecticide. 1 µL (as suggested by Biddinger et al. (2013) and Helson et al. (1994)) of each concentration was directly applied to the dorsal thorax of each bee using a Hamilton repeating dispenser. The insecticide concentrations applied were determined using a range-finding test for each compound (see table 2 for concentration ranges). Bees selected for each concentration were chosen at random to reduce any size bias. Control bees were dosed with 1 µL of pure acetone. Any bees that did not recover from CO_2 gassing after 30 minutes were excluded from the experiment. Tested individuals were placed back into cages in the incubator, with five bees per cage. Disposable plastic cages were used during pyrethroid bioassays as repeated use could result in a build-up of these insecticides within cages. Mortality was assessed after 24, 48, and 72 hours. Bees that were dead or seriously affected beyond recovery were classed as ‘dead’. Bees were fed sucrose throughout the test as described above. All bioassays were replicated three times, with ten bees tested per concentration.

Compound	Synergist	Concentration(s) (µg)
Thiacloprid	None	100
	PBO	100, 50, 25, 12.5, 6.25, 3.125, 1.5625
Imidacloprid	None	10, 1, 0.1, 0.01, 0.001, 0.0001
	PBO	0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125
Acetamiprid	None	100, 50, 25, 12.5, 6.25, 3.125, 1.563
Fupyradifurone	None	100
Tau-fluvalinate	None	20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625
	PBO	0.025, 0.0125, 0.00625, 0.003125, 0.001, 0.0004, 0.0002, 0.000125
Deltamethrin	None	10, 4, 2, 1, 0.5, 0.25, 0.2, 0.1, 0.05, 0.025, 0.01, 0.005
	PBO	0.02, 0.01, 0.005, 0.0025, 0.00125, 0.000625
Coumaphos	None	100
Chlorpyrifos	None	0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625

Table 2. Concentration ranges of insecticides applied to bees to produce dose response curve and LD_{50} values.

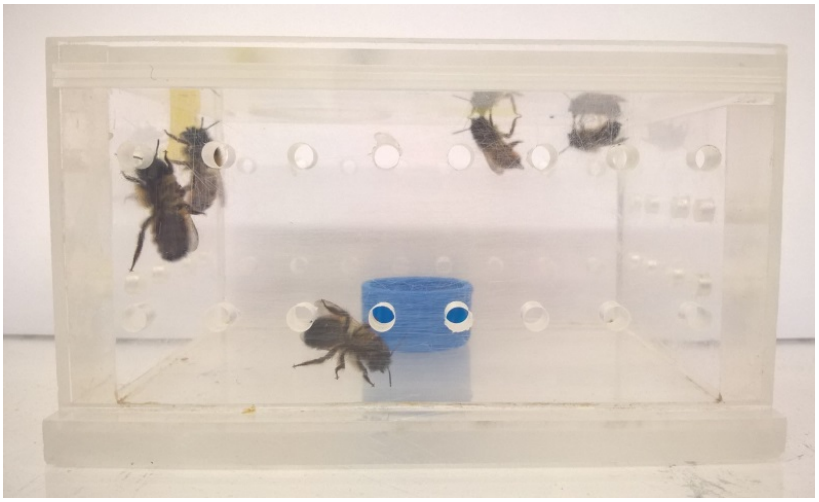


Figure 13. Set up for housing bees during bioassays and general upkeep.

3.2.3 Synergist bioassays

Synergist bioassays were carried out as described above but included the addition of the maximum sublethal dose (100 µg/bee in 1 µL) (Rinkevich et al., 2015) of PBO applied 1 hour prior to insecticide/acetone application. In addition to the acetone control each bioassay included a synergist control. Each bioassay was replicated three times, with ten bees tested per concentration (see table 2 for concentration ranges).

3.2.4 Analysis

Only bioassays with ≤10% control mortality were included in analysis. GenStat (GenStat, VSN International) was employed to carry out probit regression analysis. Abbott's formula (Abbott, 1925) was used to correct for any control mortality observed in bioassays. A generalised linear model was selected to generate dose-response curves, LD_{50} values, confidence limits and other statistical measurements. LD_{50} values were considered significantly different when 95% confidence limits did not overlap. Synergism ratios were calculated by dividing the LD_{50} values without inhibitor by the corresponding LD_{50} values with inhibitor.

3.3 Results

3.3.1 Neonicotinoids and butenolides

Topical application of thiacloprid at the highest dissolvable concentration (100 µg/bee) resulted in no observed mortality. As at least one concentration causing 100% mortality is required to generate a dose-response curve, an LD_{50} value could not be generated. Instead, a limit test was performed as recommended in the OECD guidelines in order to demonstrate that the LD_{50} of this compound is greater than 100 µg/bee. In contrast, imidacloprid was found to be 'highly toxic' to *O. bicornis* with an LD_{50} value of 0.046 µg/bee 48 hours after topical application.

When PBO was co-applied with thiacloprid the sensitivity of *O. bicornis* to this compound increased over 7-fold, but little synergism (2-fold) was observed during co-application with imidacloprid (figure 14).

Availability of bees allowed for the testing of two other 'bee friendly' insecticides, namely acetamiprid and flupyradifurone. Like thiacloprid, acetamiprid is a cyano-substituted neonicotinoid, and was also found to exhibit low toxicity (LD_{50} = 7.58 µg/bee) when topically applied. The butenolide flupyradifurone was found to be 'practically non-toxic' to *O. bicornis* when applied topically (LD_{50} >100 µg/bee).

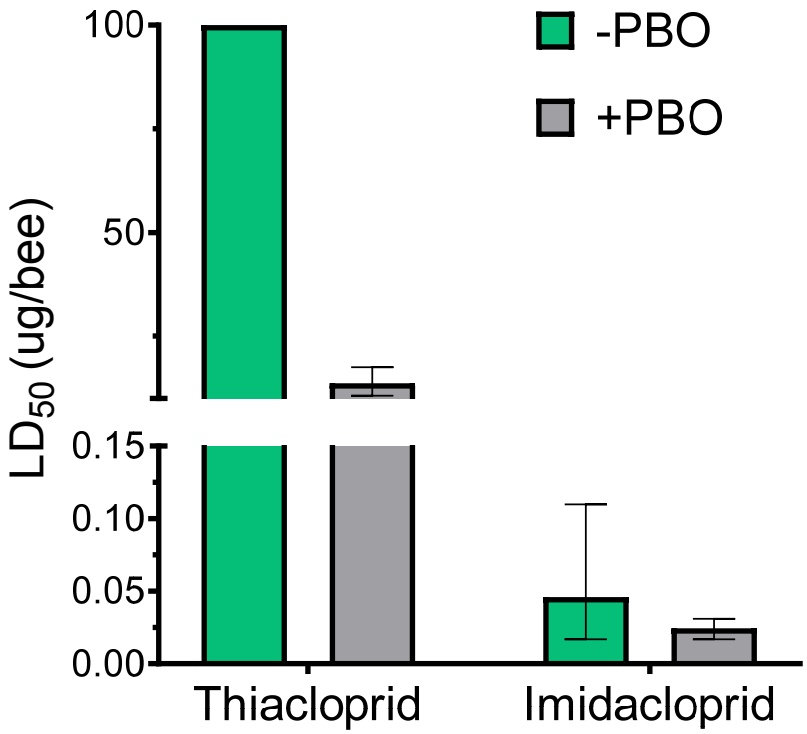


Figure 14. *O. bicornis* acute contact LD_{50} values (± 95% confidence intervals) for imidacloprid and thiacloprid 48 hours after application with and without the insecticide synergist piperonyl butoxide (PBO). Note: the LD_{50} of thiacloprid is greater than 100 µg/bee.

3.3.2 Pyrethroids

Similar to the neonicotinoids, *O. bicornis* exhibits significant differences in sensitivity to pyrethroid insecticides. Tau-fluvalinate (LD_{50} = 3.8 µg/bee) was found to be >15-fold less toxic than deltamethrin (LD_{50} = 0.25 µg/bee). Synergist bioassays revealed substantial synergism, with >580-fold increase in the toxicity of tau-fluvalinate. A lower level of synergism was observed for deltamethrin with a 190-fold increase (figure 15).

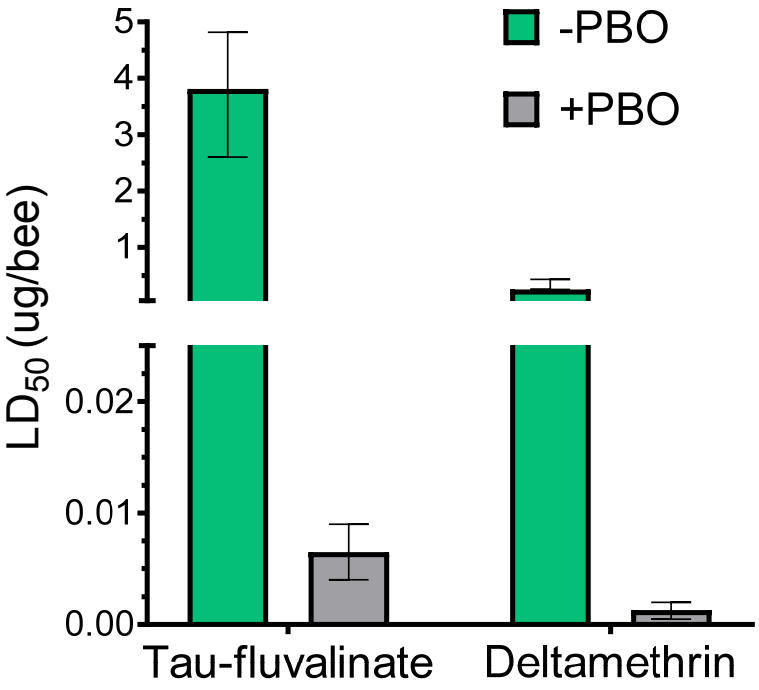


Figure 15. *O. bicornis* acute contact LD_{50} values (± 95% confidence intervals) for tau-fluvalinate and deltamethrin 48 hours after application with and without the insecticide synergist piperonyl butoxide (PBO).

3.3.3 Organophosphates

Topical application of the highest dissolvable dose of coumaphos (100 µg/bee) was found to result in no observable mortality. In the same manner as thiacloprid testing, a limit test was performed in order to demonstrate that the LD_{50} of this compound is greater than 100 µg/bee. *O. bicornis* was found to be considerably more susceptible to chlorpyrifos, with an LD_{50} of 0.09 µg/bee (figure 16).

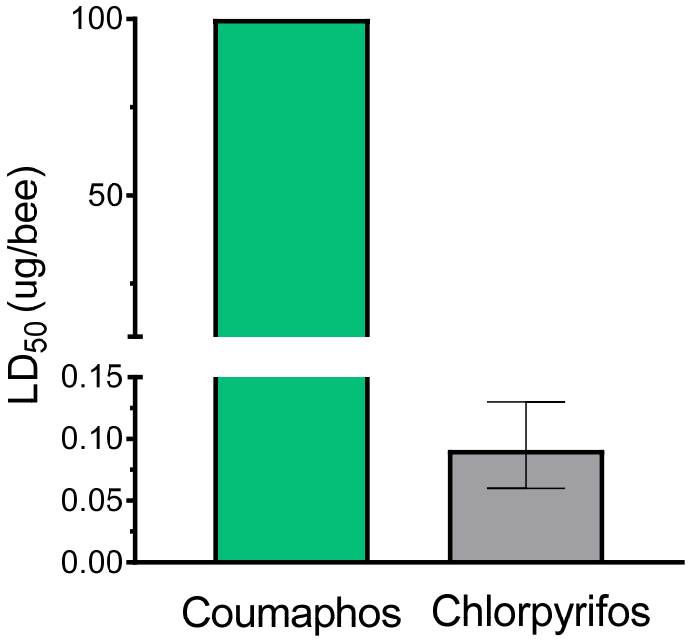


Figure 16. *O. bicornis* acute contact LD_{50} values (± 95% confidence intervals) for coumaphos and chlorpyrifos 48 hours after application. Note: the LD_{50} of thiacloprid is greater than 100 µg/bee.

Compound	Synergist	LD_{50} 48h (ug/bee)	95% CI	Slope	±SE	Synergism Ratio
Thiacloprid	None	>100	n/a	n/a	n/a	n/a
	PBO	13.69	1.03-1.25	1.6	0.19	>7.3
Imidacloprid	None	0.046	0.017-0.11	0.56	0.08	n/a
	PBO	0.024	0.017-0.032	1.7	0.51	1.9
Acetamiprid	None	7.58	5.06-10.55	1.4	0.18	n/a
Tau-fluvalinate	None	3.811	2.6-4.82	2.7	0.91	n/a
	PBO	0.0065	0.004-0.009	1.2	0.09	587
Deltamethrin	None	0.25	0.11-0.43	1.6	0.34	n/a
	PBO	0.0013	0.0005-0.002	1.6	0.36	190
Chlorpyrifos	None	0.091	0.06-0.13	2.1	0.39	n/a
Coumaphos	None	>100	n/a	n/a	n/a	n/a
Flupyradifurone	None	>100	n/a	n/a	n/a	n/a

Table 3. *O. bicornis* acute contact LD_{50} values (± 95% confidence intervals) and slope (±SE) of select insecticides 48 hours after application with and without the insecticide synergist piperonyl butoxide (PBO). Synergism ratio is also shown where determined.

3.4 Discussion

As previously shown for *A. mellifera* (Iwasa et al., 2004), *O. bicornis* also exhibits differential sensitivity to neonicotinoids (>2,000-fold difference). This differential toxicity is not confined to neonicotinoid compounds but extends to other insecticide classes including the pyrethroids and the organophosphates.

Differential neonicotinoid sensitivity was greatly suppressed on addition of the P450 inhibitor, PBO, prior to thiacloprid application, but interestingly very minor synergism was observed on co-application with imidacloprid. A similar synergistic effect was demonstrated by Iwasa et al. (2004) who found a 154-fold increase in the toxicity of thiacloprid to honey bees after PBO application but little synergistic effect on imidacloprid, with only a 1.7-fold increase. These

results provide the first line of evidence for the role of P450s in determining the sensitivity of *O. bicornis* to neonicotinoid insecticides.

Even greater synergism was observed when PBO was co-applied with tau-fluvalinate (580-fold) and to a lesser extent deltamethrin (190-fold). This synergistic effect may have been exaggerated on account of these bioassays being carried out on bees emerged towards the end of the season when bees may be weaker (Dmochowska et al., 2013) and thus more susceptible to insecticide exposure. Nevertheless, Johnson et al. (2006) also found that the toxicity of tau-fluvalinate to *A. mellifera* was greatly synergised (980-fold) on application of PBO. The magnitude of synergism of tau-fluvalinate was greater than that of deltamethrin, suggesting P450-mediated detoxification contributes to tau-fluvalinate tolerance. This finding is consistent with work on *A. mellifera*, where members of the *cyp9q* subfamily (*cyp9q1, cyp9q2, cyp9q3*) have been found to metabolise tau-fluvalinate (Mao et al., 2011). Due to its relatively low acute toxicity to honey bees, tau-fluvalinate is commonly used in-hive to treat infestations of the bee parasite *Varroa destructor* (Johnson et al., 2006).

Potential inhibition of this biochemical defence mechanism should be considered when using PBO in conjunction with insecticides in the field. The effect of PBO/insecticide mixtures on honey bees has been tested by Moores et al. (2012), who carried out a 4-day field study involving the spraying of oilseed rape crops with a tau-fluvalinate/PBO mixture. It was concluded that exposure had no detrimental effects on honey bee health. The apparent lack of synergism observed by Moores et al. (2012) compared to others may have been due to the substantially lower concentrations used (0.00024 µg/µL of tau-fluvalinate and 0.0001 µg/µL of PBO), though some mortality of *O. bicornis* (~15%) was observed during this study at exposure levels of 0.000125 µg/µL of tau-fluvalinate. Longer-term exposure to PBO/insecticide mixtures may result in the build-up of PBO to higher concentrations that could cause significant inhibition of this detoxification mechanism. Fungicides, which are frequently applied in agriculture, have also been found to inhibit P450 activity (Iwasa et al., 2004). Indeed, a number of triazole fungicides have been found to act as competitive inhibitors of P450-mediated detoxification of quercetin by *A. mellifera* (Mao et al., 2017). Additionally, during laboratory studies triflumizole, an important agricultural fungicide, was found to increase the toxicity of the cyano-substituted

neonicotinoid thiacloprid 1141-fold to *A. mellifera* (Iwasa et al., 2004), demonstrating the importance of further studies assessing the potential synergistic interactions of fungicides and *O. bicornis* P450s in the field.

Due to its low toxicity to *A. mellifera*, the organophosphate coumaphos is commonly used as an in-hive *Varroa* treatment (Calatayud-Vernich et al., 2016). Coumaphos was also found to be 'practically non-toxic' to *O. bicornis* when topically applied. As a solitary bee species, *O. bicornis* is not commonly exposed to in-hive treatments, suggesting utilisation of a pre-existing mechanism to detoxify coumaphos instead of a developed tolerance. Indeed, the reduced toxicity of this compound to *A. mellifera* has previously been attributed to metabolism by members of the cyp9q subfamily (Mao et al., 2011).

When comparing with other studies, there is considerable variation in the LD_{50} values generated. Previously reported 48h LD_{50} values of topically applied imidacloprid on *A. mellifera*, for example, range between 0.018 (Iwasa et al., 2004) and 0.2 (Biddinger et al., 2013), and 0.049- 0.1 µg/bee (Nauen et al., 2001). These studies show variation over a factor of ten and thus caution should be applied when comparing studies. This dissimilarity likely stems from variation in the methodology used by research facilities and inherent variation in the tested bees (e.g. genetic background). Nevertheless, closely related *Osmia* spp. have been found to show similar levels of insecticide sensitivity to that reported in this study. For example, acute toxicity bioassays of topically applied acetamiprid on *Osmia cornifrons* generated a similar LD_{50} value of 4 µg/bee 48 hours after application (compared to 7 µg/bee for *O. bicornis*) (Biddinger et al., 2013).

Current insecticide risk assessment guidelines only provide instruction on the testing of honey bees. Predictions of the risk of insecticides to non-*Apis* bee species are commonly based on these guidelines. Although, a number of studies have shown high variability in insecticide sensitivity among bee species (Arena and Sgolastra, 2014; Del Sarto et al., 2014), likely due to biological differences (e.g. body size). A need for risk assessment guidelines on other bee species has been identified and will soon include *Osmia* spp. During this study significant adaptation of these guidelines was carried out to enable the assessment of the acute contact toxicity of adult *O. bicornis* females, mainly due to difficulties in the feeding method. Oral tests on *O. bicornis* were also

attempted during this PhD but proved to be extremely time consuming and much more variable. Due to the small window for testing (2 months per year) the more robust topical bioassays were prioritised.

In summary, *O. bicornis* exhibits significant differences in susceptibility to insecticides within the same class. This result highlights the need for each insecticide to be treated individually when assessing its impacts on bee health, even between compounds within the same class. The differential sensitivity observed was greatly reduced on addition of the P450 inhibitor PBO, providing the first line of evidence that P450s play a role in the detoxification of some insecticides. It is important to note that the bioassays used in this study only assess the acute toxicity to bees. In order to fully assess the impact of insecticides on *O. bicornis,* long-term exposure tests measuring both lethal and sublethal affects should be carried out. Furthermore, it would be interesting to explore the existence of differential insecticide sensitivity in other life stages of *O. bicornis* e.g. the larval stage and examine how this compares with P450 expression during development.

Chapter four: Pharmacokinetics of select insecticides

Statement of contribution

The work described in this chapter was carried out during a BBSRC CASE industrial placement at Bayer CropScience (Germany). Data were collected with the assistance of Harald Koehler and Marion Zaworra.

4.1 Introduction

As detailed in section 1.5, insecticide resistance in insect pests can develop through a number of mechanisms. Knowledge of these mechanisms provides a useful basis from which the mechanism(s) utilised by bees to defend themselves against xenobiotics can be explored. This chapter describes the investigation of insecticide affinity for the target-site and rate of cuticular penetration as possible explanations for the variation in sensitivity of *O. bicornis* to different insecticides observed in chapter 3.

4.1.1 Binding affinity of insecticides targeting nicotinic acetylcholine receptors

Minor alterations to the chemical structure of a compound can cause dramatic differences in its affinity for its target receptor, leading to variation in its acute toxicity (Moffat et al., 2016). This chapter focuses on the binding affinity of insecticides targeting nAChRs, namely imidacloprid, thiacloprid, acetamiprid and flupyradifurone. As detailed in section 1.4.1, nAChRs are nervous system receptors that form ligand-gated ion channels (LGICs) comprising an integral ion channel that mediates the action of the neurotransmitter ACh (Matsuda et al., 2005). These insecticides mimic ACh, causing overstimulation and eventually death of the insect (Casida and Durkin, 2013).

The understanding of the MoA of these insecticides has been significantly advanced by the development of radiolabelled compounds (a compound in which one or more of its atoms have been replaced by a radioisotope). Tritiated imidacloprid ([^{3}H]imidacloprid) was the first radiolabelled insecticide targeting the nAChR to be produced, and was found to bind with nanomolar affinity to housefly *Musca domestica* nAChRs (Latli and Casida, 1992). Since then

[^{3}H]imidacloprid has been used extensively to explore the binding affinity of other neonicotinoid compounds by measuring their ability to displace [^{3}H]imidacloprid from its binding site (Manjon et al., 2018; Nauen et al., 2001; Nauen et al., 2015).

All neonicotinoids have been found to elicit selective agonistic activity on insect nAChRs, varying from partial, full, or super activity. This range in activity appears to be related to both the chemical structure of the compound and also to the subtype (transcriptional isoform) of nAChRs expressed in target cells (Tan et al., 2007). For example, the binding affinity of neonicotinoids on nAChRs expressed by thoracic ganglia of the American cockroach *Periplaneta americana* was found to vary with compound structure. Neonicotinoids with a heterocyclic ring (i.e. thiacloprid and imidacloprid) were found to exhibit partial agonistic actions, whereas open-chained neonicotinoids (i.e. acetamiprid) were much more effective agonists (Tan et al., 2007). This agonist efficacy has been found to impact insecticidal action (Liu et al., 1993; Nishiwaki et al., 2000). Differential binding ability of nitro- and cyano- substituted neonicotinoids has been demonstrated in *A. gossypii*. Clones of this species containing the R81T nAChR mutation were found to show greater resistance to nitro-substituted neonicotinoids such as imidacloprid compared to cyano-substituted neonicotinoids such as thiacloprid (Hirata et al., 2017).

Pharmacological characterisation of the honey bee nAChR revealed that imidacloprid is a partial agonist of nAChRs in dissociated honey bee Kenyon cells (Déglise et al., 2002), selectively binding to a single binding site (ACh recognition site) (Tomizawa et al., 1995). Like imidacloprid, other neonicotinoids have been found to exhibit nanomolar binding affinity for the [^{3}H]imidacloprid binding site in insects (Wiesner and Kayser, 2000). Electrophysiological and radioligand displacement studies revealed that the butenolide flupyradifurone also acts as a partial agonist of *M. domestica* nAChRs, with nanomolar binding affinity (IC_{50}=2.38$\pm$1.93nM) (Nauen et al., 2015).

The aim of this chapter was to assess whether the differential sensitivity of *O. bicornis* to neonicotinoids was due to differences in the binding affinity of these compounds to nAChRs. This was investigated by performing [^{3}H]imidacloprid displacement assays.

4.1.2 Cuticular penetration ability of select insecticides

The cuticle is the outermost part of the insect body and forms the initial barrier against external foreign substances (Balabanidou et al., 2018). The cuticle of an insect is composed of two main layers; a thin outer epicuticle, containing lipids and proteins, and a thicker procuticle, containing proteins and chitin. The composition of the cuticle varies greatly during the life stages of an insect, and is controlled by a complex genetic system. Recent sequencing of the honey bee genome found a reduced number of genes coding for cuticular proteins compared to *Drosophila, Anopheles* and *Tribolium* genomes, although it is thought that the size of this cuticular proteome will increase as more genes are characterised (Kucharski et al., 2007).

The ability of an insecticide to penetrate through the insect cuticle and reach its target-site is dependent on a number of factors, including the cuticle composition and the chemical structure of an insecticide. Variation in the molecular structure of a compound can be accompanied by changes in intermolecular forces, affecting properties such as volatility and solubility (Hadaway, 1971).

Isotopic labelling has provided valuable insights into the penetration ability of insecticides by enabling the tracking of their permeation through the cuticle and into the insect. A reduced rate of insecticide penetration through the cuticle has been found to play a role in insecticide resistance of a number of different pest species. For example, neonicotinoid resistance of the aphid *Myzus persicae* was found to be partly due to reduced cuticular penetration. After 50 hours, 78% of applied [^{3}H]imidacloprid was found to have penetrated through the cuticle of susceptible clones whereas only 44% had penetrated in resistant clones (Puinean et al., 2010).

To date, there have been no studies looking at the differential ability of nitro- and cyano-substituted neonicotinoids to penetrate through the insect cuticle. Thus, the second aim of this chapter was to assess whether differences in the sensitivity of *O. bicornis* to neonicotinoids is due to differences in the speed of cuticular penetration by looking at the rate of internalisation of radiolabelled

imidacloprid and thiacloprid. Two additional ‘bee friendly’ compounds were also tested, namely acetamiprid and flupyradifurone.

4.2 Methods

4.2.1 Binding affinity of insecticides targeting nicotinic acetylcholine receptors

10 g of snap frozen (-80°C) adult female *O. bicornis* heads were homogenised in 200 mL of ice-cold potassium phosphate buffer (see appendix 1) using a motor-driven Ultra Turrax blender (IKA). The homogenate was centrifuged at 1,200 g for 10 minutes at 4°C, and the resulting supernatant was filtered through five layers of cheesecloth. Protein concentration was determined using Bradford reagent (Sigma) and BSA (Sigma) as a reference as described in section 2.10. Assays were performed in 96-well plates with bonded GF/C filter membranes (PerkinElmer). Binding assays were carried out in a total volume of 1 mL, consisting of 850 µL of homogenate (200-300 µg), and 50 µL of [^{3}H]imidacloprid (25,000 disintegrations per minute (dpm)) in potassium phosphate butter. After 5 minutes, 100 µL of unlabelled imidacloprid was added to the assay at the following concentrations: 1,000, 100, 10, 3, 1, 0.3, 0.1, 0.01, 0 nM. The assay was then incubated at 22°C, with shaking, for 60 minutes. Samples were filtered through pre-wetted Whatman GF/C glass fibre filters and rinsed three times with potassium phosphate buffer. The filter was then dried at 55°C for 40 minutes. After 16 hours the bound radioactivity was quantified using a liquid scintillation analyser (Perkin Elmer Tri-Carb 2910 TR). To do this, filters were placed into 5 mL plastic scintillation vials and covered with 3 mL of scintillation fluid cocktail (Ultima Gold, PerkinElmer). All binding studies consisted of three replicates and IC_{50} values (concentration of unlabelled ligand displacing 50% of [^{3}H]imidacloprid from its binding site) were calculated using a 4-parameter logistic non-linear curve fitting routine (Graphpad, prism). IC_{50} values were considered significantly different when 95% confidence limits did not overlap. The method described above was also used to assess the binding affinity of thiacloprid, acetamiprid and flupyradifurone.

4.2.2 Cuticular penetration ability of select insecticides

Prior to testing, compound application error was evaluated by applying tested compounds to a series of ten filter papers and measuring the average amount applied as described below. Bees were emerged as described in section 3.2.1. 24-hour old adult female *O. bicornis* were anaesthetised with CO_2 for 5-10 seconds to allow application of the compound. 5,000 dpm/µL of [^{14}C]imidacloprid (synthesised by Bayer CropScience) was applied to the dorsal thorax of each bee using a Hamilton repeating dispenser. Three replicates of groups of five bees were placed into cages and fed a 50% sucrose solution from vertically hanging 2 mL syringes. Control bees were treated with acetone. The [^{14}C]imidacloprid was rinsed off of each group of bees at different time intervals (0, 2, 4 and 24 hours after application) with 1 mL of 90% acetone three times. The amount of internalised [^{14}C]imidacloprid was measured by individually combusting the acetone-washed bees at 900°C in house at Bayer CropScience in an Ox 120c oxidizer (Harvey Instruments Co., USA) followed by liquid scintillation counting of the released $^{14}CO_2$. The level of excreted [^{14}C]imidacloprid and/or metabolites was estimated by wiping used cages with filter papers dipped in acetone and measuring the radioactivity. Six filter papers were used per cage. 0.5 mL aliquots of cuticular rinse or individual filter papers were added to 3 mL of scintillation fluid cocktail in 5 mL plastic scintillation vials and the radioactivity was quantified using a liquid scintillation analyser. The method described above was also used to assess the penetration ability of [^{14}C]thiacloprid, [^{14}C]acetamiprid and [^{14}C]flupyradifurone (synthesised by Bayer CropScience). The average percentages of compound recovered from wash solution/ penetrated at each time point was calculated using Excel. Total penetration was calculated by the addition of internal and excreted amounts. To compare the penetration abilities of each compound a one-way ANOVA was used.

4.3 Results

4.3.1 Binding affinity of insecticides targeting nicotinic acetylcholine receptors

Radioligand binding studies revealed that imidacloprid, thiacloprid, acetamiprid and flupyradifurone all bind with nanomolar affinity to nAChRs of *O. bicornis* head membrane preps. No significant differences were found in the binding affinity of these compounds (table 4).

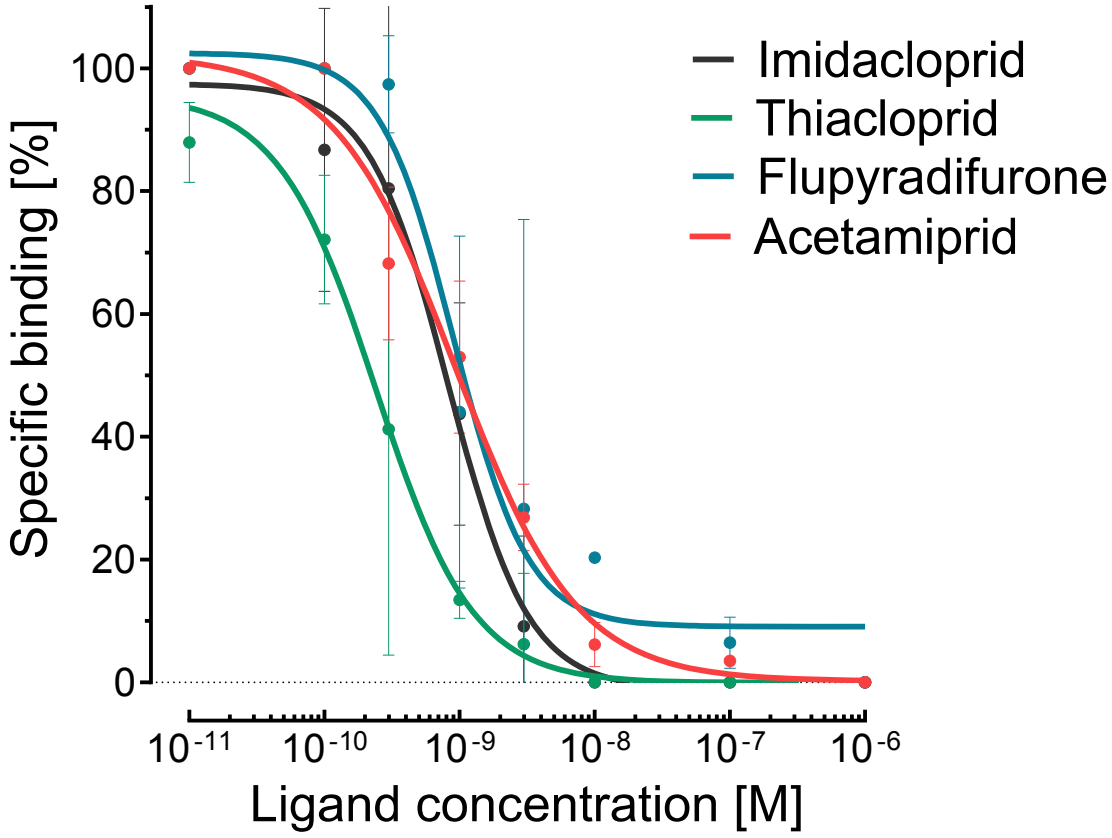


Figure 17. Specific binding affinity of imidacloprid, thiacloprid, flupyradifurone and acetamiprid displacing [H^3]imidacloprid at nicotinic acetylcholine receptors in *O. bicornis* head membrane preparations. Data points are mean values (n=2 ±95% CIs).

Compound	IC_{50} (M)	95% CI
Imidacloprid	0.844	-0.996-97.51
Thiacloprid	0.231	-0.068-95.63
Flupyradifurone	0.911	-9.073-102.50
Acetamiprid	0.938	0.212-102.20

Table 4. Imidacloprid, thiacloprid, flupyradifurone and acetamiprid IC_{50} values ($\pm$ 95% confidence limits) calculated using a 4-parameter logistic non-linear curve fitting routine.

4.3.2 Cuticular penetration ability of select insecticides

For all four compounds, the majority of penetration occurred in the first 4 hours post-application, with a gradual increase in internal recovery over 24 hours. Similar levels of total penetration were seen for thiacloprid (30%) imidacloprid (27%) and flupyradifurone (14%). A greater level of total penetration was observed for acetamiprid (48%) although this result was not significant (figures 18 and 19, ANOVA, $F_{3,12}$= 0.43, p= 0.73).

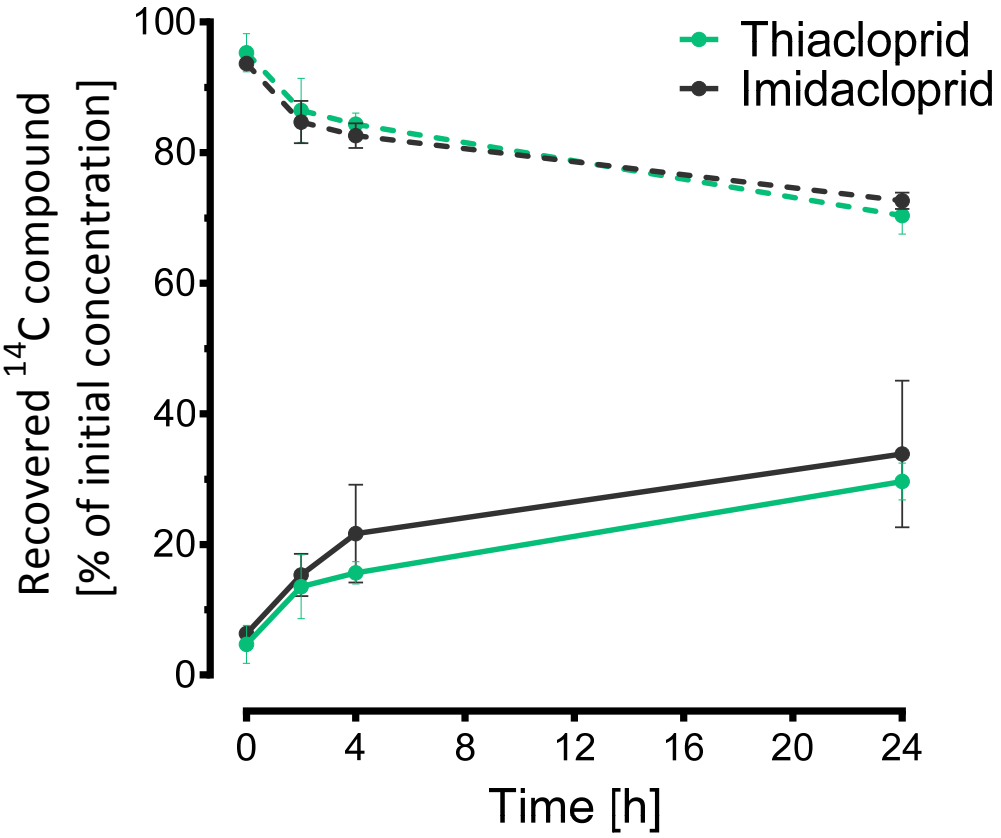


Figure 18. Total percentage of initial ^{14}C equivalent imidacloprid and thiacloprid penetrated through the cuticle (solid line) and percentage of initial ^{14}C equivalent imidacloprid and thiacloprid recovered from rinse solution (dashed line) over 24 hours. Data points are mean values (n=3 ±SD).

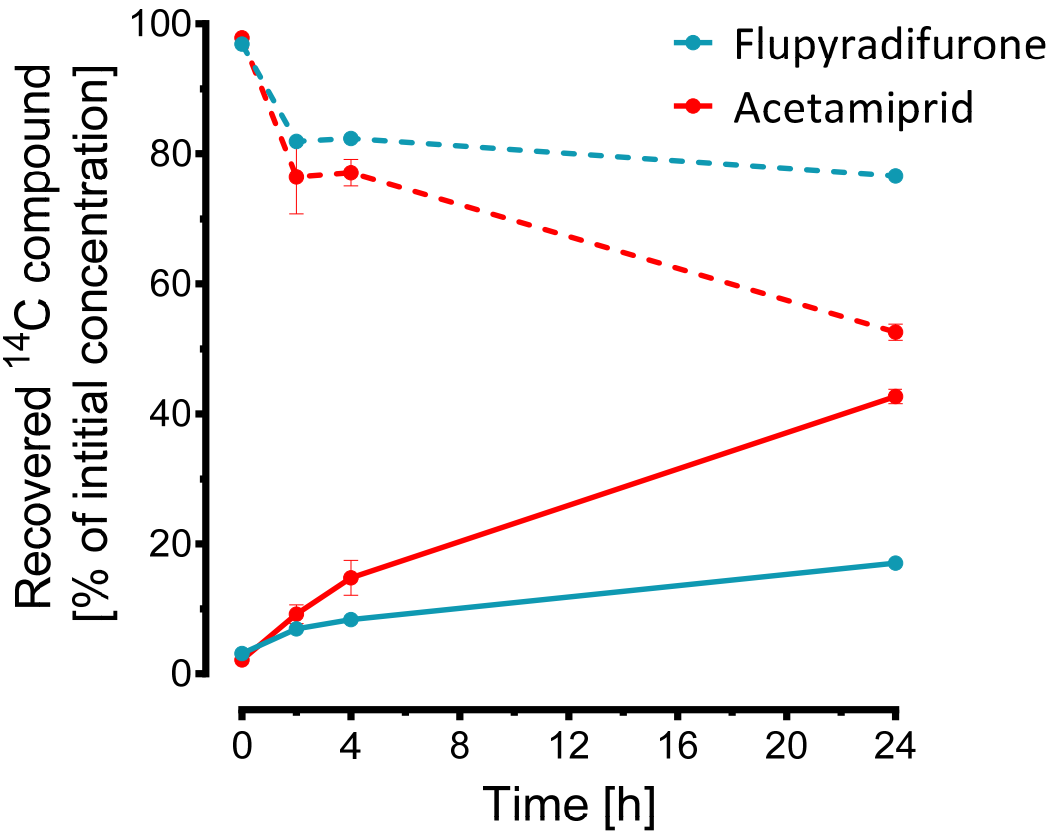


Figure 19. Total percentage of initial ^{14}C equivalent acetamiprid and flupyradifurone penetrated through the cuticle (solid line) and percentage of initial ^{14}C equivalent acetamiprid and flupyradifurone recovered from rinse solution (dashed line) over 24 hours. Data points are mean values (n=3 ±SD).

4.4 Discussion

4.4.1 Binding affinity of insecticides targeting nicotinic acetylcholine receptors

Radioligand competition assays conducted with a well-known nAChR agonist ([^{3}H]imidacloprid) revealed no significant differences in the binding affinity of imidacloprid, thiacloprid, acetamiprid and flupyradifurone to *O. bicornis* nAChRs isolated from head membrane preparations.

This finding is consistent with a previous study which performed [^{3}H]imidacloprid competition assays on *A. mellifera* and *B. terrestris* nAChRs and observed no differences in binding affinity of imidacloprid and thiacloprid (*A.*

mellifera; IC_{50} of 1.2 and 1.1 nM for imidacloprid and thiacloprid respectively, *B. terrestris*; IC_{50} of 0.71 and 0.62 nM for imidacloprid and thiacloprid respectively) (Manjon et al., 2018). These results demonstrate that differences in the binding affinity of imidacloprid and thiacloprid for nAChRs does not explain the marked variation in bee sensitivity to these compounds.

Furthermore, the 'bee friendly' insecticides acetamiprid and flupyradifurone exhibited similar binding abilities to imidacloprid and thiacloprid (0.94 and 0.91 M respectively), further demonstrating that the binding affinity of these insecticides to the receptor target does not play a role in their efficacy.

4.4.2 Cuticular penetration ability of select insecticides

The role of cuticular penetration ability in differential neonicotinoid bee sensitivity was explored by comparing the amount of penetrated [^{14}C]imidacloprid vs. [^{14}C]thiacloprid after 24 hours. Similar levels of penetration were found for both compounds, demonstrating that reduced penetration ability of thiacloprid is unlikely to be a contributing factor to the variation seen in the sensitivity of *O. bicornis* to neonicotinoids.

Even though acetamiprid and thiacloprid are both cyano-substituted neonicotinoids, topical insecticide bioassays described in chapter 3 revealed that *O. bicornis* is substantially more susceptible to acetamiprid compared to thiacloprid (LD_{50} of 7.58 µg/bee and LD_{50} of >100 µg/bee respectively). As well as cyano- and nitro- substituted compounds, neonicotinoids can be divided into two other subgroups: those equipped with a heterocyclic ring (e.g. imidacloprid and thiacloprid), and those containing an open-chain electronegative pharmacophore (e.g. acetamiprid) (Tan et al., 2007). The greater ability of [^{14}C]acetamiprid to penetrate through the cuticle of *O. bicornis* could be due to its acyclic, open-chained structure, resulting in the greater efficacy observed during topical bioassays. To investigate this further, studies would need to be performed with similarly-structured compounds (e.g. clothianidin, dinotefuran and nitenpyram).

Of the compounds tested, the butenolide flupyradifurone exhibited the lowest

penetration ability, with only 14% of the applied compound penetrated after 24 hours, reflecting its bee-safe profile and low toxicity observed during topical bioassays (LD_{50} of >100 µg/bee).

Insecticide sensitivity of bees is greatly increased when insecticides are ingested orally compared to when applied topically (e.g. Manjon et al., (2018)), consistent with the cuticle acting as a partial barrier against contact exposure. Indeed, during this study a maximum of 48% of the initially applied compound was seen to penetrate through the cuticle (though further penetration may have occurred after 24 hours). The penetration ability of insecticides can also be assessed by comparing oral and topical toxicity data (e.g. Puinean et al., (2010)) and similar approaches could be used in future studies to confirm the findings of this chapter. For some insects, the penetration of insecticides actually begins in appendages such as the legs. Thus, some studies have applied insecticide to surfaces rather than directly onto insect bodies to resemble a more realistic exposure route (Balabanidou et al., 2018).

In summary, variation in the susceptibility of *O. bicornis* to neonicotinoids observed in chapter 3 is unlikely to be due to differences in either binding affinity or the penetration ability of these compounds. Instead, the penetration ability of these compounds may to be related to whether the electronegative pharmacophore moiety is cyclic (imidacloprid/thiacloprid) or acyclic (acetamiprid).

Chapter five: Assembly and comparative exploration of the *O. bicornis* CYPome

Statement of contribution

Sample preparation for transcriptome/genome sequencing was carried out by Bartek Troczka. Transcriptome/genome assembly was carried out by Kumar Saurabh Singh and thus will only be described in brief. Manual annotation and curation of the transcriptome/genome, PCR validation, and phylogenetic analyses were carried out as part of this PhD. Manual annotation and curation of additional bee species was carried out by Kumar Singh, Angie Hayward, Bartek Troczka and myself. Verified P450 sequences were sent to David Nelson (leader of the P450 naming committee) to be officially named according to figure 10 in section 1.5.3.1.

5.1 Introduction

Synergist bioassays detailed in chapter 3 demonstrated that *O. bicornis* females pre-treated with the P450 inhibitor PBO were 7-fold more sensitive to thiacloprid but no more sensitive to imidacloprid, suggesting that P450s play an important role in determining the sensitivity of *O. bicornis* to thiacloprid.

The first bee genome to be sequenced was that of the western honey bee, *A. mellifera*, and provided primary insights into the CYPome of bees. One of the most notable features was the considerably lower number of P450 genes (just 47) identified relative to other insects (Claudianos et al., 2006). It was originally speculated that this reduced inventory of detoxifying genes resulted in an increased sensitivity of *A. mellifera* to insecticides compared to other insects, but this was later disproved by Hardstone and Scott (2010), who carried out an extensive meta-analysis on the sensitivity of *A. mellifera* to insecticides in comparison to other insects. Since then a number of other bee genomes have been sequenced, including the genomes of solitary bee species.

The involvement of P450s in the detoxification of xenobiotics by bee pollinators was first suggested by Iwasa et al. (2004) who found a 154-fold increase in the toxicity of thiacloprid to honey bees after the P450 synergist PBO was applied during contact bioassays. Since then, a number of P450s, predominantly belonging to the CYP3 clade, have been characterised as important detoxification genes both in bees and other insect species. Indeed, *A. mellifera* has been shown to use P450s to detoxify a number of both natural and synthetic xenobiotics. For example, CYP6AS (CYP6AS1, CYP6AS3, CYP6AS4, CYP6AS10) enzymes have been found to be involved in the metabolism of quercetin, a naturally-occurring flavonoid found in nectar and subsequently honey (Mao et al., 2009), although their catalytic activity is low compared to the activity of lepidopteran phytochemical-metabolising CYP6 enzymes. Similarly, the CYP9 family has been found to contribute to the detoxification of synthetic insecticides. Mao et al. (2011) demonstrated the multifunctional activity of CYP9Q1, CYP9Q2 and CYP9Q3 which are able to metabolise both the pyrethroid tau-fluvalinate, and the organophosphate coumaphos, although their subsequent metabolites were not quantified. A recent study by Manjon et al. (2018) further characterised the CYP9Q subfamily by identifying their role in the breakdown of neonicotinoids, specifically thiacloprid and to a lesser extent imidacloprid into their hydroxylated forms. This study went on to characterise the functional orthologs of these genes in the buff-tailed bumble bee *Bombus terrestris* and identified CYP9Q4 and CYP9Q5 as metabolisers of thiacloprid. To date, the detoxification genes utilised by other bee species have not been identified.

Prior to the commencement of this PhD, no genomic resources were available for *O. bicornis.* Thus, the sequencing of a transcriptome (the complete set of expressed RNA transcripts in an organism (Zhang et al. 2012)) was necessary in order to identify the specific P450s involved in thiacloprid metabolism. As previously mentioned, P450s belonging to the CYP3 clade have most commonly been implicated in the detoxification of insecticides (Feyereisen, 2006). As such, members of the bee toxicogenomics team (of which this PhD project is part of) functionally expressed the entire CYP3 clade of *A. mellifera,* identifying a single P450, CYP9Q3, as a highly efficient metaboliser of thiacloprid, likely explaining why thiacloprid is relatively non-toxic to honeybees

(see Manjon et al. (2018)). This provided a starting point from which potential thiacloprid-detoxifying orthologs could initially be identified from the *O. bicornis* transcriptome. However, some transcriptome sequences were only partial and rapid amplification of cDNA ends (3' RLM-RACE), a wet biology technique used to obtain full-length cDNA, yielded limited results. In recent years, next-generation sequencing has improved dramatically in both efficiency and cost (Ansorge, 2009), which allowed the first draft genome of *O. bicornis* to be sequenced, providing a more complete coverage of the *O. bicornis* CYPome.

In addition to identifying candidate thiacloprid-detoxifying P450s, the sequencing of the genome allowed for further exploration of the evolution of P450s involved in xenobiotic detoxification, by comparing the *O. bicornis* CYPome with the publically available CYPomes of 11 other solitary and eusocial bee species.

5.2 Methods

5.2.1 Transcriptome assembly

O. bicornis adults were emerged and maintained as described in chapter 3. Bees were snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. RNA was individually extracted from three female and three male bees as described in section 2.2. DNA quality and quantity were assessed by spectrophotometry using a Qubit assay (ThermoFisher Scientific) and gel electrophoresis as described in section 2.8.1. RNA was used as a template for the generation of barcoded libraries using the TrueSeq RNA library preparation kit (Illumina) and sequenced across two lanes of an Illumina HiSeq2500 flowcell (100 bp paired end reads). To ensure deep coverage a single female bee was sequenced across one lane and to examine differential gene expression, three female bees and three male bees each with a unique barcode were multiplexed for sequencing across a second lane. Sequencing was carried out by The Genome Analysis Centre (TGAC). The quality of the raw data was assessed by FastQC v0.11.7 software (Babraham Bioinformatics) and trimmed using TrimGalore v0.4.2 (Babraham Bioinformatics). Transcriptome assembly was carried out using the Trinity Software Suite (Grabherr, 2011). The genome of

the most closely related bee species available, *Megachile rotundata,* was used as a reference during assembly.

5.2.2 Manual annotation and curation of a transcriptome

The software used in assembly can be error-prone, and thus manual annotation of genes was necessary (Feyereisen, 2006). A provisional list of candidate P450 genes was created by manual annotation and curation of transcripts using Geneious v8.1.3 software (Biomatters Ltd). Briefly, candidate P450 nucleotide sequences were compared to the NCBI non-redundant sequence database (Geer et al., 2010) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The sequence of the resulting top hit was imported into Geneious and aligned with the candidate P450 nucleotide sequence to identify the 3' and 5' ends of the sequence. Any sequences that did not generate a P450 hit were excluded. Pseudogenes were identified when more than one error (i.e. in-frame stop codon, unmatched intron splice site) was observed. The gene length and orientation was noted and each sequence was given a unique gene number.

5.2.3 PCR verification

All candidate P450 genes were verified by PCR. RNA was extracted from a pool of 3-5 female bees as described in section 2.2. First-strand cDNA was synthesised at a concentration of 200 ng/μL as described in section 2.3. Typical PCR reactions were carried out as described in section 2.4, with adaptation of annealing temperatures depending on primers used. PCR products were visualised on a 1% agarose gel as described in section 2.6. PCR products were purified as described in section 2.8 and sent for sequencing as detailed in section 2.9. Validation of P450 sequences was carried out by aligning sequencing results of each PCR product with the original transcripts from the transcriptome using Geneious (figure 20).

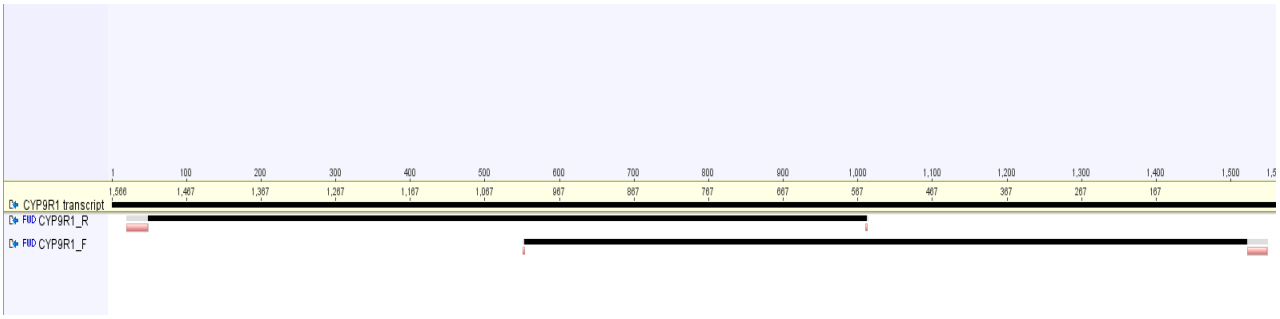


Figure 20. Example of a Geneious pair-wise alignment of sequenced PCR fragments with original gene transcript (*cyp9r1* transcript) from the transcriptome used to PCR validate sequences. CYP9R1_F PCR fragment was produced from sequencing with a forward primer and CYP9R1_R PCR fragment was produced from sequencing with a reverse primer. The top bar shows the scale of sequence length.

5.2.4 Genome assembly

O. bicornis adults were emerged and maintained as described in chapter 3. Bees were snap frozen in liquid nitrogen and stored at -80°C prior to DNA extraction. Total DNA was extracted from a single haploid male using the E.Z.N.A® Insect DNA kit (Omega Bio-Tek) following the manufacturers protocol. Prior to extraction bees were checked for the presence of mites in order to prevent DNA contamination. DNA quality/quantity was assessed by spectrophotometry using a Qubit assay (ThermoFisher Scientific) and gel electrophoresis as detailed in chapter 2.6. Based on the statistics obtained from Genomescope, DISCOVAR *de novo* –v52488 genome assembler was used to assemble the genome. To increase the contiguity of the assembled genome, long mate-pair sequencing was carried out with insert sizes of 450, 2, 4.2, 8.5, 9.5 and 11.5k, enabling scaffolding of data and improvement of the N50 (statistic parameter of contig/scaffold length). Default DISCOVAR *de novo* settings were used with untrimmed raw sequences to generate a first pass assembly (named v1). Redundans –v 0.12a (Pryszcz and Gabaldon, 2016). Default parameters were used to scaffold assembly v1 using both paired-end and mate-paired libraries in ascending order along with their average insert sizes. Sequences <1000 bp were excluded from further assembly steps. Gene prediction was carried out using BRAKER-v 2.1.0 (Hoff et al., 2016). Once assembled, the Benchmarking Universal Single-Copy Orthologues v3 (BUSCO) pipeline (Simão et al., 2015) was used to assess the completeness of the gene space in the assembled genome with >99% of Insecta and Arthropoda test genes identified as complete in the assembly (see table 5 for results).

5.2.5 Manual annotation and curation of the *O. bicornis* genome

A BLAST database was created using Geneious comprising all of the previously curated and PCR validated transcriptome sequences. Genome sequences were BLAST searched against this database in order to match genes based on sequence percentage identity. Novel genes were given a unique gene number. The validated sequences were then sent to David Nelson (leader of the P450 naming committee) to be officially named according to figure 10 in section 1.5.3.1.

5.2.6 Manual annotation and curation of other bee CYPomes

P450 nucleotide sequences from the genomes of 11 bee species (*Apis mellifera, Apis dorsata, Apis florea, Apis cerana, Bombus terrestris, Bombus impatiens, Megachile rotundata, Habropoda laboriosa, Eufriesea Mexicana, Dufourea novaeangliae* and *Melipona quadrifasciata*) were downloaded from the NCBI sequence database. As detailed in section 5.2.2, genome sequences were BLAST searched against the NCBI non-redundant sequence database to confirm correct annotation. Any sequences that did not generate a P450 hit were excluded. Pseudogenes were identified when more than one error (i.e. in-frame stop codon, unmatched intron splice site) was observed. The gene length and orientation was noted and each sequence was given a unique gene number. They were then sent to David Nelson (leader of the P450 naming committee) to be officially named according to figure 10 in section 1.5.3.1.

5.2.7 Phylogenetic analysis

All phylogenetic analyses were carried out using Geneious. Molecular evolutionary genetic analysis (MEGA-X) v6 (Tamura, 2013) was used to determine the optimal algorithm to use when producing phylogenetic trees. Prior to sequencing the genome of *O. bicornis*, curated full-length P450s from the *O. bicornis* transcriptome were used to identify orthologs of *A. mellifera* CYP9Q3. Specifically, a multiple protein alignment was carried out in Geneious with *O. bicornis* P450s and *A. mellifera* P450s. This alignment was then used to produce a tree using the UPGMA method, also in Geneious (Netherlands, 2008). Once the genome was assembled this analysis was repeated to include the final *O. bicornis* CYPome based on a combination of transcriptome and genome data. Reference honey bee P450s used in phylogenetic analyses were downloaded from NCBI. Phylogenetic comparison of the 12 bee species was carried out using a PHYML substitution model Le Gascuel (LG) with 1,000 bootstrap replicates.

5.3 Results

Genome sequencing yielded 548,590,674 reads. Data were assembled to generate an *O. bicornis* genome size of 212.9 Mb, consistent with the predicted genome size of 143.5 Mb. The final assembly consisted of 10,223 scaffolds with a scaffold and contig N50 of 604 kb and 303 kb respectively. Manual annotation and curation of P450 transcripts revealed that the CYPome of *O. bicornis* comprises 52 putatively functional genes. Nine of these genes were not identified from transcriptome assembly, namely *cyp6as131*, *cyp6as132*, *cyp6bd1*, *cyp9p22*, *cyp9p24*, *cyp9p2*, *cyp303a1*, *cyp306a1*, and *cyp314a1*. Similar to other insect species, these P450s fall into four phylogenetically distinct clades (CYP2, CYP3, CYP4 and mitochondrial clades), of which the CYP3 clade (comprising 33 P450s) is by far the largest (figure 21). Phylogenetic comparison of the CYP9 family of *O. bicornis* with that of 11 other bee species revealed that *O. bicornis,* as well as most other solitary bee species, lacks the CYP9Q subfamily that has been implicated in neonicotinoid detoxification in eusocial bee species (figure 22). The most closely related subfamily to this in *O. bicornis* was found to be CYP9BU, a novel subfamily not identified in other annotated bee genomes. This subfamily comprises two P450 genes (*cyp9bu1* and *cyp9bu2*) and appears to share a recent common ancestor with the CYP9Q subfamily. Following this, CYP9R is the next most closely related subfamily (represented by *cyp9r1*, *cyp9r38* and *cyp9r39*), and is also present in *A. mellifera* (figure 21). These five P450s were initially selected for further characterisation as detailed in chapter 6. Following functional characterisation of these P450s, a second group of candidate P450s were selected for functional characterisation. This selection was based on the available closely-related full-length P450s obtained from the transcriptome as the genome had not been sequenced at this point. This group consisted of *cyp6as151, cyp336a36, cyp336a35, cyp9dn1, cyp6aq55, cyp336l1* and *cyp6as127*.

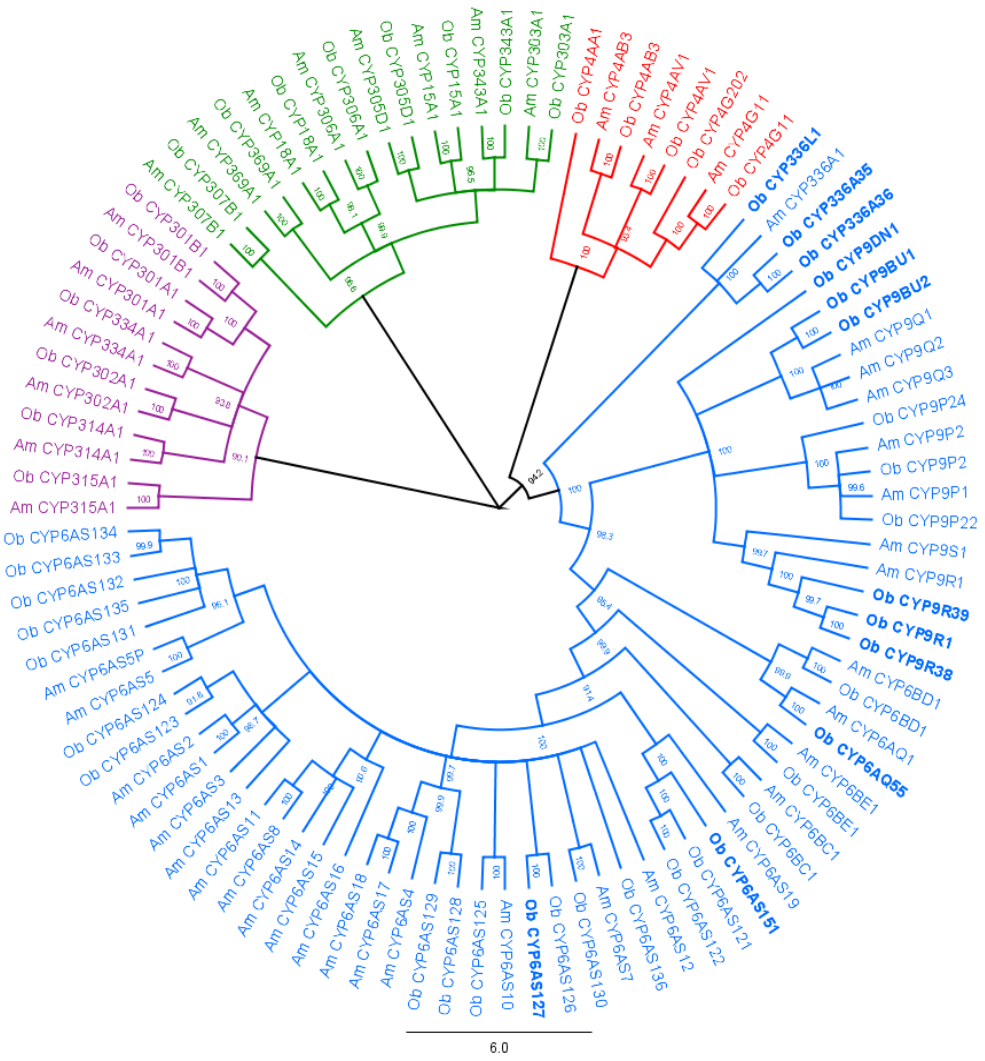
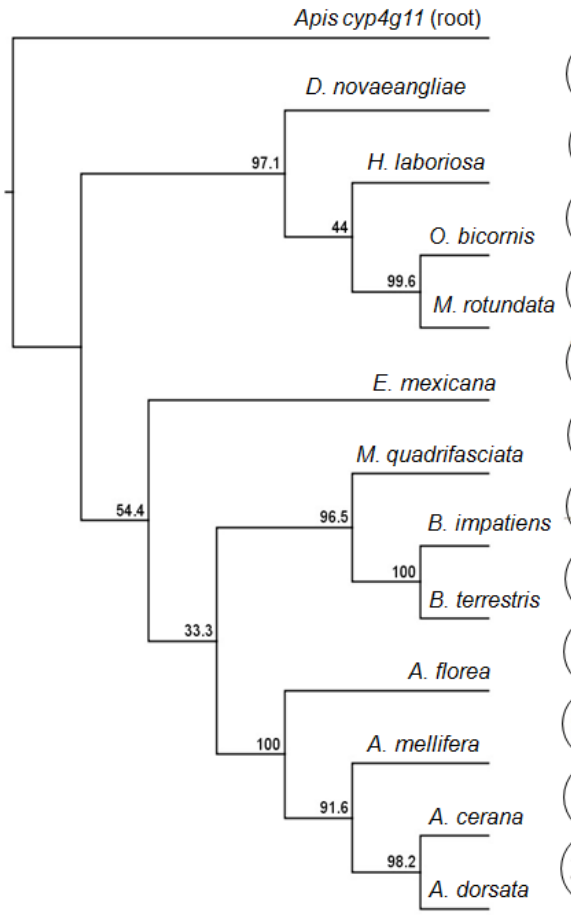


Figure 21. Phylogenetic tree with bootstrap values comparing the *Apis mellifera* (Am) and *Osmia bicornis* (Ob) CYPomes. Colours indicate different P450 clades: blue CYP3, purple mitochondrial, green CYP2, and red CYP4. P450s selected for further characterisation are marked in bold.

Clade	% BUSCO	#BUSCO	Complete BUSCO	Single copy	Duplicated	Fragmented	Missing
Eukaryota	99.4	303	301	296	5	0	2
Arthropoda	99.9	1066	1065	1059	6	1	0
Insecta	99.7	1658	1652	1646	6	4	2

Table 5. Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis of the O. bicornis genome



Sociality	Total no. of P450s	No. of CYP9 members	No. of CYP9Q members	No. of CYP9R members	No. of CYP6 members	No. of CYP6AS members	No of CYP6AQ members
Ancestrally solitary	45	7	0	2	17	10	4
Ancestrally solitary	38	8	1	2	15	8	1
Ancestrally solitary	52	9	0	3	21	17	1
Ancestrally solitary	49	8	0	3	21	13	3
Obligate complex eusocial	45	8	2	3	19	15	1
Facultative simple eusocial	54	8	2	3	25	20	1
Obligate simple eusocial	47	5	3	1	23	11	8
Obligate simple eusocial	49	7	3	1	21	12	6
Obligate complex eusocial	35	3	1	1	17	13	1
Obligate complex eusocial	46	7	3	1	22	18	1
Obligate complex eusocial	41	7	3	1	18	15	1
Obligate complex eusocial	42	6	3	1	17	13	1

Figure 22. Phylogeny of the 12 bee species analysed in this study. Represented by the CYP9R1 protein sequence of each species. Phylogenetic tree was produced using a PHYML substitution model Le Gascuel (LG), 1,000 bootstrap branch support, rooted with *Apis mellifera* CYP4G11. Bootstrap values are shown in the figure. The table details the level of sociality, total number of P450s, and number of CYP9, CYP9Q, CYP9R, CYP6, CYP6AS and CYP6AQ members present in the CYPome of each bee species.

	Am CYP9Q3	Ob CYP9BU1	Ob CYP9BU2	Ob CYP9R1	Ob CYP9R38	Ob CYP9R39
Am CYP9Q3		49.035%	47.852%	43.605%	43.023%	40.734%
Ob CYP9BU1	49.035%		60.000%	43.822%	44.015%	41.618%
Ob CYP9BU2	47.852%	60.000%		42.406%	43.195%	40.079%
Ob CYP9R1	43.605%	43.822%	42.406%		95.745%	66.667%
Ob CYP9R38	43.023%	44.015%	43.195%	95.745%		66.667%
Ob CYP9R39	40.734%	41.618%	40.079%	66.667%	66.667%	

Figure 23. Heat map comparing the percentage sequence identity of the first round of candidate thiacloprid-detoxifying P450s with *Apis mellifera* CYP9Q3.

	Am CYP9Q3	Ob CYP6A...	Ob CYP6A...	Ob CYP6A...	Ob CYP9DN1	Ob CYP33...	Ob CYP33...	Ob CYP33...
Am CYP9Q3		29.787%	30.528%	28.655%	37.094%	27.875%	27.875%	24.756%
Ob CYP6AQ55	29.787%		34.843%	34.118%	28.488%	29.273%	27.308%	26.719%
Ob CYP6AS127	30.528%	34.843%		48.810%	31.238%	29.644%	27.668%	26.877%
Ob CYP6AS151	28.655%	34.118%	48.810%		29.412%	29.921%	29.134%	26.523%
Ob CYP9DN1	37.094%	28.488%	31.238%	29.412%		26.931%	25.545%	26.535%
Ob CYP336A35	27.875%	29.273%	29.644%	29.921%	26.931%		65.269%	52.191%
Ob CYP336A36	27.875%	27.308%	27.668%	29.134%	25.545%	65.269%		51.394%
Ob CYP336L1	24.756%	26.719%	26.877%	26.523%	26.535%	52.191%	51.394%	

Figure 24. Heat map comparing the percentage sequence identity of the second round of candidate thiacloprid-detoxifying P450s with *Apis mellifera* CYP9Q3.

Figure 25. Proximity of CYP9BU1, CYP9BU2, CYP9R1 and CYP9R38, CYP9R39 on scaffold 00060

5.4 Discussion

It has previously been speculated that the reduced number of detoxification genes in *A. mellifera* compared to other insect species is due to a collective 'social immune system', resulting in reduced exposure to environmental

stressors such as xenobiotics and thus a decreased reliance on genome-encoded detoxification genes (Johnson et al., 2018; Wilson-Rich et al., 2009). However, consistent with other studies (e.g. Johnson et al. (2018)), the CYPomes of *O. bicornis* and other solitary bee species were found to contain a similarly reduced number of P450s and thus eusociality *per se* does not seem to explain the reduced P450 inventory (Berenbaum and Johnson, 2015). These results suggest that like humans (Zanger and Schwab, 2013), *O. bicornis* and other bee species rely upon a comparatively small number of generalist enzymes to detoxify xenobiotics. In particular, these bee species appear to have a reduced number of P450s belonging to the CYP4 clade (between four and five members) compared to *D. melanogaster* (22 members) (Tijet et al., 2001b), suggesting that these P450s are of less importance. Instead, the majority of the CYPome falls into the CYP3 clade. These 'blooms' are thought to be reflective of responses to selective agents such as phytochemicals in the environment (Berenbaum, 2002). The CYP3 clade largely consists of the CYP6 and the CYP9 families. Within the CYP6 family the CYP6AS subfamily appears to be over-represented in terms of number of genes, with 17 members in the case of *O. bicornis*, and is unique to species in the order Hymenoptera, (Mao et al., 2009). This over-representation was found to be conserved throughout the bee species analysed, suggestive of an important functional role. Indeed, as mentioned previously CYP6AS enzymes have been found to be key metabolisers of quercetin and thus this duplication is thought to have occurred due to exposure to naturally-occurring xenobiotics (Mao et al., 2009). Additionally, a similar study by Johnson et al. (2018) analysed the CYPomes of ten bee species including the eusocial carnivorous *Polistes* species and concluded that the positive selection on CYP6AS genes in *Apis* and *Bombus* but not *Polistes* suggests that the CYP6AS subfamily was an important facilitator of the shift from carnivory to florivory in bees. During this PhD, two members of the CYP6AS subfamily (CYP6AS152 and CYP6AS127) were characterised (see chapter 6).

The CYP9R subfamily was also found to be present among all 11 of the bee species analysed. The size of the CYP9R subfamily seems to vary between species, with solitary bee species tending to have a higher number of CYP9R members compared to eusocial bee species. Furthermore *B. terrestris* and *B.*

impatiens seem to have a particularly high number of CYP6AQ P450 members and thus characterising these P450s could prove interesting.

Within the CYP3 clade, the CYP9 P450 family is also well preserved among bee species, indicative of an important functional role. The CYP9Q subfamily, which has been found to play an important role in the detoxification of xenobiotics, was found to be restricted to eusocial species, with the exception of *H. laboriosa* which has a single CYP9Q gene (*cyp9q9*). The absence of the CYP9Q subfamily in solitary bees suggests that this subfamily diverged after the evolution of eusociality from a solitary ancestor (Kapheim et al., 2015), and thus may play a detoxification role specific to eusocial bees (Kapheim et al., 2015; Mao et al., 2013). It could be expected that without this xenobiotic-detoxifying subfamily *O. bicornis* would be more susceptible to neonicotinoids, which could have wide implications for bee health. However, as described in chapter 3, *O. bicornis* exhibits similar levels of sensitivity to thiacloprid as that of *A. mellifera* and *B. terrestris* (Manjon et al., 2018), and this sensitivity was found to increase when *O. bicornis* females were pre-treated with the P450 inhibitor PBO. Therefore it is likely that *O. bicornis* employs other P450s belonging to a different subfamily for the detoxification of cyano-substituted neonicotinoids. The most closely related subfamilies to *A. mellifera* CYP9Q are CYP9BU followed by CYP9R and thus members of these families were initially selected for further characterisation. The CYP9BU subfamily is a novel subfamily found to be exclusive to the *O. bicornis* CYPome. The close proximity of these five orthologs on the scaffold and their lack of introns suggests that these genes are a result of a rapid and recent duplication of an initially retrotransposed gene (figure 25).

Overall, sequencing of the *O. bicornis* transcriptome and subsequently the genome revealed that the *O. bicornis* CYPome contains a similar number of P450s to that of other bee species. The completeness of the genome assembly (as shown by BUSCO data) and the similarity of P450 abundance to other bee species makes it highly likely that all *O. bicornis* P450s were identified within this study. Interestingly, solitary bee species were found to lack the CYP9Q subfamily found in eusocial bee species, which has previously been implicated in detoxification of a range of xenobiotics. As *O. bicornis* is no more sensitive to thiacloprid than *A. mellifera* it is likely that *O. bicornis* employs P450s from a

different subfamily to detoxify neonicotinoids. The availability of the *O. bicornis* genome provides the genomic resources for a wide range of future studies, which is of particular importance due to the inclusion of *O. bicornis* in EFSA risk assessment guidelines of insecticides on bee pollinators.

Chapter six: Functional analysis of candidate P450s

Statement of contribution

The transgenic *Drosophila* studies detailed in this chapter were carried out with the teaching assistance of Christoph Zimmer, Manu Eckel-Zimmer, Rafael Homem and Bartek Troczka.

6.1 Introduction

P450s have been found to exhibit a number of functional roles, including biosynthesis, activation, and catabolism (Feyereisen, 2012). The role of P450s in the detoxification of insecticides by insect pests is well characterised (Panini et al., 2016), however, much less is known about the functional roles of P450 enzymes in bee pollinators. If P450s do play a role in determining their sensitivity to insecticides, then this knowledge could be leveraged to safeguard bee health. This chapter details the first functional characterisation of P450 enzymes in a solitary bee species, aiming to determine the contribution of P450-mediated metabolism to the sensitivity of *O. bicornis* to neonicotinoids. Additionally, the ability of P450s to metabolise naturally-occurring alkaloids was assessed.

A number of systems have previously been utilised to functionally express P450s, including *E. coli*, *Drosophila melanogaster,* yeast and insect cell lines (Homem and Davies, 2018; Lu et al., 2010). In this chapter *O. bicornis* candidate detoxification genes identified in chapter 5 were expressed *in vitro* in an insect cell line, and *in vivo* by the production of transgenic *Drosophila* lines expressing P450 genes of interest. Additionally, incubation of insecticides and alkaloids with whole microsomal fractions containing multiple P450 enzymes was used to provide initial insights into the global P450 activity of *O. bicornis*.

6.1.1 Microsomes

Microsomal preparation from insect tissue homogenates remains the most widely used preliminary step in the functional characterisation of insect P450s (Feyereisen, 2012). Microsomes are artificial structures of reformed

endoplasmic reticulum that form during tissue homogenisation and contain a high concentration of multiple P450 enzymes (Vrbanac and Slauter, 2013). Incubation of microsomal preparations with xenobiotics provides a primary insight into the metabolic ability of a species, and the overall P450 activity (Feyereisen, 2012). For example, Suwanchaichinda and Brattsten (2001) explored the induction of P450s in *Aedes albopictus* larvae by extracting microsomes from pentachlorophenol (PCP)-treated larvae and measuring the yield of O-demethylation of methoxyresorufin.

6.1.2 Metabolism of alkaloids by *O. bicornis*

As discussed in section 1.4.4, alkaloids are a group of naturally-occurring secondary plant metabolites (from hereon SPMs) that serve as a chemical defence against herbivorous insects (Johnson et al., 2010). These alkaloids are predominantly found in the leaves of plants but can also be found in the pollen and nectar, subsequently exposing bee pollinators to these potentially harmful compounds (Detzel and Wink, 1993). The defence mechanism(s) utilised by bees to metabolise these compounds remains largely uncharacterised, but there is substantial evidence that both honey bee adults and larvae are able to efficiently metabolise nicotine, a SPM produced by *Nicotiana tabacum* (tobacco) (du Rand et al., 2017a; du Rand et al., 2017b). This metabolism is thought to be carried out by P450 enzymes belonging to the CYP9Q subfamily (Rand et al., 2015), which have also been implicated in neonicotinoid metabolism (Manjon et al., 2018). Indeed, P450s have been found to be involved in the metabolism of nicotine in insect pest species. Overexpression of CYP6CY3 (a highly efficient metaboliser of nicotine) for example, has allowed the host plant shift of *Myzus persicae* to tobacco (tobacco-adapted race *Myzus persicae nicotianae*) (Bass et al., 2013).

Like *A. mellifera*, *O. bicornis* is a generalist pollinator species (Sedivy et al., 2011) and thus is likely to be exposed to an array of SPMs whilst foraging, yet their ability to detoxify these secondary metabolites is unknown. The aim of this study was to explore the capability of *O. bicornis* microsomes and recombinant P450 enzymes to detoxify select alkaloids. As neonicotinoids are based on the chemical structure nicotine, it was expected that members of the CYP9 family

would exhibit cross-resistance of SPM and neonicotinoid insecticides, as was the case for *M. persicae nicotianae* (Bass et al., 2013).

6.1.3 Expression of P450s in an insect cell line

Expression of individual P450s using the baculovirus expression system is a widely employed technique for the production of microsomal P450s (Feyereisen, 2012), including the successful expression of a number of insect P450s (e.g. Zimmer et al., (2014); Gong et al., (2017)), including bee P450s (Manjon et al., 2018).

The baculovirus expression system most frequently uses two commercially available insect cell lines, namely Sf9 and High Five™ insect cell lines. These lines originate from the ovarian tissues of *Spodoptera frugiperda* (fall army worm) (Vaughn et al., 1977) and *Trichoplusia ni* (cabbage looper) (Granados et al., 1994) respectively. Heterologous gene expression relies on the infection of these cells with a baculovirus, which are naturally occurring viruses that specifically infect insects. Due to their very late gene expression system, baculoviruses are highly suitable candidates for foreign gene expression and have been found to yield high levels of recombinant protein (van Oers, 2011, Lu et al., 2010).

Cytochrome P450 reductase (CPR) is essential for P450 catalysis, transferring electrons from NADPH to the P450 heme centre. Thus, co-infection of cells with baculoviruses carrying the P450 of interest and baculoviruses carrying CPR is necessary for the successful expression of microsomal P450s. CPRs are coded for by a single gene, and are highly conserved between insects (Feyereisen, 2012). In this case, house fly (*Musca domestica*) CPR was utilised in the expression of *O. bicornis* P450s due to its prior success in expression of other insect P450s (Zimmer et al., 2014). Cytochrome b_5 has also been shown to play a role in P450 catalysis, but is not vital for sufficient P450 expression (Feyereisen, 2012).

Michaelis-Menten kinetics is commonly used to characterise enzymatic

reactions using functionally expressed genes, generating comparable kinetic parameters such as the maximum velocity of a reaction (V_{max}) and the binding affinity of the substrate for the enzyme (K_m), which can be defined as half of the concentration of substrate needed to produce a reaction velocity that is half of V_{max} (Obach and Reed-Hagen, 2002).

Various techniques have been developed to measure the activity of expressed P450s. For example, Liquid Chromatography-Mass Spectroscopy (LC-MS) is a technique that enables both the separation of several components within a liquid and the identification of their chemical structure (Pitt, 2009). LC-MS has been used to explore insect metabolism of a number of different insecticides by measuring the depletion of parent compounds or the formation of metabolites (Manjon et al., 2018). However, LC-MS can be time consuming and assessing P450 activity using fluorescent P450 model substrates can be a quicker and more cost-effective method, allowing the rapid testing of multiple samples in parallel. In these assays P450s oxidise a low fluorescent model substrate into a high fluorescent metabolite. These products can then be directly measured using a fluorescence microplate reader (Cheng et al., 2009). A number of coumarin- and resorufin-derived model substrates have been developed to 'map' the catalytic competence and substrate specificity of P450s (Feyereisen, 2012; Inceoglu et al., 2009), with P450s found to exhibit substantial variation in their preferences for different model substrates (e.g. Nakamura et al. (2001)).

HPLC-MS has been used to identify the main metabolites produced from biotransformation of ingested imidacloprid by *A. mellifera*. Namely, 4/5-hydroxy-imidacloprid (OH-imidacloprid), followed by 4,5-dihydroxy-imidacloprid, 6-chloronicotinic acid (6-CNA), and olefin and urea derivatives. These metabolites are formed through three major metabolic routes. The first involves the hydroxylation of the imidazolidine ring, forming OH-imidacloprid and further 4,5-dihydroxy-imidacloprid. The second concerns the oxidative cleavage of the imidacloprid methylene bond followed by oxidation to form 6-CNA. The third involves removal of the nitro group followed by oxidation to form urea derivatives. The route of metabolism of olefin is not established, but is thought to result from dehydration of OH-imidacloprid (Suchail et al., 2004). Both OH-imidacloprid and olefin have been found to exhibit some toxicity to honey bees

when orally ingested whereas 6-CNA does not. Likewise, radioligand competition assays and electrophysiology studies showed that OH-imidacloprid and olefin act antagonistically on neurons isolated from the antennal lobe and bind with nanomolar affinity to *A. mellifera* nAChRs. 6-CNA was not found to elicit any displacement of [^{3}H]imidacloprid from its binding site, with the conclusion that the toxicity of these metabolites seems to depend on the retention of the nitroguanidine pharmacophore during biotransformation (Nauen et al., 2001). Less is known about the biotransformation of thiacloprid, but the main metabolites include 5-hydroxy-thiacloprid (OH-thiacloprid) and 6-CNA (EPA, 2003).Topical bioassays of honey bees using OH-thiacloprid revealed that this metabolite is 'practically non-toxic' to honey bees (LD_{50} value of >100 µg/bee) (Manjon et al., 2018).

In this chapter the catalytic activity of the candidate P450s selected in chapter 5 was explored using both LC-MS analysis and P450 model substrate fluorescence assays, with a focus on the hydroxylation of the neonicotinoids imidacloprid and thiacloprid. This was carried out to explore whether variation in the metabolic rate of imidacloprid and thiacloprid hydroxylation can explain the observed variation in the sensitivity of *O. bicornis* to these insecticides.

6.1.4 Expression of P450s in transgenic *Drosophila*

For over a century, the fruit fly *D. melanogaster* has been used extensively to study a broad range of biological phenomena. The success of this model organism is due to a number of factors, including its short life cycle, comparatively small genome size, and ease of rearing in the laboratory (Aquilina and Cauchi, 2018).

Genetic modification of *D. melanogaster* using the GAL4/UAS system, along with other genomic approaches such as RNA interference and most recently CRISPR/Cas9, has proven to be a useful tool in the functional characterisation of individual genes involved in insecticide resistance (Homem and Davies, 2018; Perry and Batterham, 2018). The GAL4/UAS system involves two components that are maintained in separate parental lines: a female driver line, which contains a promoter and GAL4 (a protein derived from brewer's yeast

(*Saccharomyces cerevisiae*), normally used as a regulator of genes induced by galactose), and a male responder line, which contains an upstream activating sequence (UAS) and the gene of interest. In this case, transgenes were expressed under the control of an actin promoter, which expresses GAL4 throughout the insect. Upon crossing of these lines, GAL4 is produced and binds to the UAS, activating the transcription and expression of the transgene in the progeny. The inactivity of the parental lines means that the transgene is not continuously expressed, and thus genes coding for toxic products can be expressed successfully (Duffy, 2002).

Pioneering work by Daborn et al. (2002) used the GAL4/UAS system to identify a single P450 (CYP6G1) responsible for DDT resistance in *D. melanogaster.* Since then, the GAL4/UAS system has been utilised to validate the role of a number of other genes in the insecticide resistance of pest species (Daborn et al., 2012). For instance, expression of CYP6ER1 in *D. melanogaster* was found to be sufficient in conferring resistance to imidacloprid, confirming the role of CYP6ER1 overexpression in imidacloprid resistant brown planthoppers (*Nilaparvata lugens*) (Pang et al., 2016).

During this study the role of select P450s in the sensitivity of *O. bicornis* to neonicotinoids was explored by comparing the resistance of fly lines containing P450s with the resistance of fly lines with the same genetic background minus the transgene (control) to neonicotinoids. Additionally, the accumulative metabolic effect of three P450s was explored by producing transgenic lines expressing CYP9BU1, CYP9BU2, and CYP9R38 located on the same chromosome.

6.2 Methods

6.2.1 Expression of P450s in an insect cell line

6.2.1.1 Initiation of insect cell lines from frozen stocks

All handling of insect cell lines was performed in a sterile laminar flow hood (Astec-Microflow) using aseptic techniques. 1 mL stocks of Sf9 and High Five™

cells (Invitrogen) at a concentration of approximately 1x 10^{-7} and 3 x10^{-6} cells/mL respectively, suspended in a freezing medium (60% Grace's Insect Medium, 30% FBS and 10% DMSO or 42.5% conditioned Express Five® SFM, 42.5% fresh Express Five® SFM, 10% DMSO and 5% FBS respectively), were stored in a cryogenic dewar containing liquid nitrogen. In order to initiate new cultures, cells were placed in a 37°C water bath until almost completely thawed. The entirety of the tube was slowly dispensed into a T-25 treated tissue culture flask (CytoOne) containing 4 mL of either Sf-900™ II SFM (Gibco™) or Express Five™ SFM (Gibco™) room-temperature media. The flask was gently rocked in vertical and horizontal motions to evenly spread the cells and placed in a 27°C incubator (Sanyo) for 30-45 minutes to allow the cells to attach to the surface of the flask. Attachment was confirmed by visual inspection under a microscope. The medium was expelled to remove any dead cells, cellular debris or DMSO found in the freezing medium. This was replaced with 5 mL of fresh room temperature media. Flasks were placed back in the incubator for 24 hours or until confluency was reached. At this point, cells were passaged.

6.2.1.2 Passaging of insect cell lines

6.2.1.2.1 Adherent cultures

Media was removed from confluent T-25 flasks and 4 mL of fresh room-temperature Sf-900™ II SFM or Express Five™ SFM media was added. Cells were detached by tapping the sides of the flask. Dislodgement of cells was confirmed by visual inspection under a microscope. 1 mL of detached cells was transferred to a new T-25 flask containing 4 mL of fresh media. Cells were evenly spread as described above. Cell cultures were passaged once confluence was reached (every 2-3 days for Sf9 cells and around 2 days for High Five™ cells). Cell cultures were scaled up by dispensing 3 mL of detached cells into a T-75 flask containing 12 mL of fresh media.

6.2.1.2.2 Suspension cultures

Prior to the subculturing of suspension cultures, the cell density and viability of cultures was assessed as follows: 10 µL of cells were mixed with 30 µL of water

and 10 µL of 0.4% Trypan Blue (Gibco™) (1:5 dilution) in a 1.5 mL microcentrifuge tube. 10 µL of the resultant solution was loaded onto a Neubauer Chamber (Marienfeld) and placed under a microscope. The number of cells within the counting grid was determined using a tally counter. Cells that had taken up the Trypan Blue stain were considered non-viable and so were not included in the count. The following calculation was used to determine the cell density:

Number of cells per mL= Number of cells x 5 x 10,000

This was repeated three times and the average cell density was calculated. The cell density was used to estimate when Sf9 and High Five™ cell cultures were reaching the end of the log phase (~$3x10^{-6}$cells/mL) and thus required passaging for optimal health. Routinely, Sf9 and High Five™ cell cultures were passaged at a density of $0.4x10^{-6}$ and $1x10^{-6}$ cells/mL respectively in 30 mL of media and placed on a shaking platform (110rpm) (ThermoFisher Scientific) in a 27°C incubator. The cell density of cultures was also calculated prior to experiments to determine the volume of culture needed for seeding log phase cells into plates. High Five™ cells are commonly found to aggregate in cultures, making accurate counting of cells more difficult. To prevent this, heparin sodium salt (Sigma) was added at a concentration of 10U/mL to non-experimental cell cultures.

6.2.1.3 Synthesis of candidate P450 genes

Candidate genes were synthesized *in vitro* by GeneArt (Life Technologies) and codon optimised for *S. frugiperda*. These genes were delivered as plasmids in pDONOR221 vectors (flanking sequences 5' attB1 [ACTTTGTACAAAAAAGCAGGCT] and 3' attB2 [ACCCAGCTTTCTTGTACAAAG]) with kanamycin resistance. In order to generate sufficient plasmid stocks, genes were transformed into XL10 gold ultra-competent *E. coli* (Agilent) and purified as described in section 2.7 and 2.8.2 respectively.

6.2.1.4 Transfection of candidate genes into the baculovirus genome and generation of P1 viral stock

The BaculoDirect™ Baculovirus Expression System (Life Technologies) was used to perform an LR recombination reaction, transferring the gene of interest from the entry clone into the baculovirus genome. 1 µL of entry clone (300 ng) and 5 µL of 1X TE buffer, pH 8.0, was added to the BaculoDirect™ Linear DNA (300 ng) vial provided with the kit. 4 µL of LR Clonase™ II Enzyme Mix was added and the sample was mixed by tapping the side of the tube. The tube was then incubated for 18 hours in a 25°C water bath.

Successful recombination was confirmed by PCR using Dreamtaq mastermix as detailed in section 2.4. PCR reaction temperature cycling conditions were as follows: 95°C for 5 minutes, followed by 25 cycles of 94°C for 45 seconds, 52°C for 1 minute, and 72°C for 1.5 minutes, with a final elongation step at 72°C for 10 minutes. 25 µL PCR reactions contained a 2 µL aliquot of the LR reaction and baculovirus polyhedrin forward and V5 reverse primers (see appendix 2). PCR products of an expected size of around 1,700 bp were examined by gel electrophoresis as detailed in section 2.6.

8×10^{-5} log phase Sf9 cells ($1.5 – 2.5 \times 10^{-6}$ cells/mL) with >95% viability were seeded into each well of a six-well treated plate (Falcon) with two wells prepared for each sample. Cells were evenly distributed and allowed to attach to the plate for 15-30 minutes at room temperature. For each transfection

sample, the following solutions were prepared in 1.5 mL microcentrifuge tubes: Transfection mixture A; 8 µL of Cellfectin II reagent (Life Technologies) and 100 µL of Grace's insect medium (Gibco), Transfection mixture B; 20 µL LR recombination reaction and 200 µL Grace's insect medium. Transfection mixtures A and B were then combined and mixed gently by tapping the side of the tube. The tube was then incubated for 25-35 minutes at room temperature. After incubation the transfection mixture was divided into the two prepared wells for each transfection sample. Cells were incubated at 27°C for 5 hours. After 5 hours the transfection mixture was removed and replaced with 2 mL of fresh Sf9 media. Recombinant Baculovirus was selected for by the addition of 100µM ganciclovir (InvivoGen) to each well. The plate was placed into a sealed plastic bag containing moist paper towels in order to prevent evaporation. Plates were placed in a 27°C incubator for 72 hours or until signs of viral infection (e.g. detached or misshapen cells). Inclusion of two control wells containing cells that were not infected with a virus enabled the visual comparison of infected vs. uninfected cells.

Post-transfection, the P1 viral stock was harvested by transferring 2 mL of media from each well into sterile 15 mL Falcon tubes. Tubes were then centrifuged at 3,500 rpm for 10 minutes. The supernatant was transferred into a fresh 15 mL tube and 5% fetal bovine serum (Sigma) was added. P1 viral stocks were stored at 4°C, protected from light.

6.2.1.5 P2 viral stock

8 $x10^{-5}$ log phase Sf9 cells (1.5 – 2.5 $x10^{-6}$ cells/mL) with >95% viability were seeded into each well of a six-well treated plate, with two wells prepared for each sample. Each well contained 100 µM of ganciclovir and 10 µg/mL of gentamycin (Sigma). Cells were evenly distributed and allowed to attach to the plate for 15-30 minutes at room temperature. 10 µL of the P1 viral stock was added dropwise to each well. The plate was placed into a sealed plastic bag that contained moist paper towels to prevent evaporation. The plate was then placed in a 27°C incubator for 72 hours or until visual signs of viral infection. After 72 hours, the P2 virus was collected and stored in the same way as described above.

6.2.1.6 Viral quantification by Enzyme-Linked Immunosorbent Assay

In the following section the components of the materials written in italic can be found in table 6. P2 and P3 baculovirus titers were determined by carrying out an Enzyme-linked immunosorbent assay (ELISA) on infected Sf9 cells (adopted from BacPAK™ Baculovirus Rapid Titration Kit, Clontech) as follows:

A TC-treated 96-well plate (CytoOne) was seeded with 200 µL of log phase cells (>95% viability) at a concentration of 3.25 x10^{-5} cells/mL, with 12 wells seeded per virus. The plate was then placed in a 27°C incubator for 30-40 minutes to allow the cells to attach. Attachment was confirmed by visual inspection under a microscope. In a separate 96-well plate, viral stock dilutions were prepared by adding 10 µL of virus to 90 µL of Sf9 media, producing a 10^{-1} dilution. This was used to create a serial dilution of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Samples were mixed thoroughly between dilutions. Once the cells had attached to the plate, the media was aspirated from each well, taking care not to scrape the cell monolayer. 25 µL of the viral dilution was added to the corresponding well (see figure 26 for plate set up). Media was not removed from uninfected negative controls wells. The plate was then incubated for 50 minutes at 27°C on a shaking platform (110 rpm).

	C		10^{-3}		10^{-4}		10^{-5}		10^{-6}		
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 26. 96-well plate set up of viral titer quantification.

After 50 minutes the virus-containing media was removed and 50 µL of fresh media was added to each well. The plate was placed in a sealed plastic bag containing wet tissue and incubated for 43-47 hours at 27°C. After incubation, 150 µL of ice-cold *formyl buffered acetone* was added and the plate was incubated for 10 minutes at room temperature. The plate was then emptied and washed by adding 200 µL of *PBS + TWEEN® 20* and incubated for 5 minutes at room temperature, with shaking. This was repeated three times. 50 µL of *diluted goat serum* was added to each well and incubated for 5 minutes at room temperature, with shaking. The plate was emptied and 25 µL of *gp64 antibody* was added to each well. This was then incubated for 25 minutes at 37°C, with shaking. The antibody was dispelled and the plate was washed twice as described above. 50 µL of *Goat-anti-mouse/HRP conjugate* was added to each well and incubated for 25 minutes, with shaking. The plate was then emptied and washed three times as detailed above. 50 µL of KPL TrueBlue™ peroxidase substrate (Seracare) was added to each well and incubated for 2 hours. The number of stained blue foci/foci forming units in each well was counted using a microscope. From this, the following calculation was used to estimate viral titer:

Titer (pfu/mL)= average number of foci x dilution factor x 40 (normalization factor) x2

Name	Components
Diluted goat serum	480 µL normal goat serum 13.8 mL phosphate buffered saline (PBS) (Sigma) 6.9 µL TWEEN® 20 (Sigma)
Anti-gp64 antibody	6 µL primary mouse monoclonal Anti-gp64 antibody (Abcam) 2994 µL diluted goat serum (1:500)
Goat anti-mouse IgG antibody	10 µL polyclonal goat anti-mouse antibody/HRP Conjugate (Abcam) 4990 µL diluted goat serum (1:500)
PBS + TWEEN® 20	200 mL PBS 100 µL TWEEN® 20
Formyl buffered acetone	5.4 mL PBS 4.5 mL 37% formaldehyde solution (Sigma) 8.1 mL acetone (Sigma)

Table 6. Components of materials written in italic in viral titer quantification methods section. Volumes are sufficient for one 96-well plate.

6.2.1.7 P3 viral stock

2 x10^{-6} log-phase Sf9 cells/mL with >95% viability were seeded into a 250 mL flask (Corning) containing 50 mL of Sf9 medium. P2 baculovirus stock was added to the culture at a multiplicity of infection (MOI) of 0.1-0.2 (calculated from viral titer assays described above). Flasks were placed in a 27°C incubator on a shaking platform (110 rpm). 4 days post-infection onwards, the viability of the cells was assessed as described in section 6.2.1.2.2. When 80-90% of the cells were non-viable the P3 virus was harvested as described above (normally 7 days post-infection).

6.2.1.8 Expression of recombinant P450s

2 x10^{-6} log-phase High Five™ cells/mL with >95% viability were seeded into a 250 mL flask containing 80 mL of fresh Express Five medium. Iron (III) citrate (Aldrich) and aminolevulinic acid hydrochloride (Sigma) were added to the flask at a concentration of 0.1 µM. Viral infection at the correct MOI is vital for achieving optimal protein expression. Thus, cells were co-infected with P3 stocks of baculovirus containing the P450 and baculovirus containing house fly CPR at three different MOI ratios (3:0.5, 2:0.5 and 1:0.5 of CYP:CPR) for each P450 in order to identify optimal conditions. The house fly CPR P3 stock was kindly supplied by Christoph Zimmer (Syngenta). The infected cells were placed in an incubator at 27°C for 24 hours, with shaking. After 24 hours iron (III) citrate and aminolevulinic acid hydrochloride were added to each flask at a final concentration of 0.1 mM. Cultures were incubated for another 36 hours, after which the cells were harvested by differential centrifugation. In brief, 50 mL of the cell culture was transferred to a 50 mL Falcon tube and centrifuged at 1,000 rpm for 5 minutes at 15°C. The supernatant was discarded and the remaining culture (30 mL) was added to the Falcon tube. The centrifugation step was repeated and the supernatant discarded. The resultant pellet was then resuspended in 30 mL of homogenisation buffer (see appendix 1). Cells were broken up using a digital sonicator (Branson) at 50-60% amplitude, pulsing for 2 of every 4 seconds for 1 minute. The solution was centrifuged at 1,500 g for 10 minutes at 4°C. The supernatant was then transferred to an ultracentrifuge tube and centrifuged at 100,000 g for 1 hour at 4°C. The supernatant was discarded

and the resulting microsomal pellet was resuspended in 1-5 mL of Buffer R (see appendix 1) using a glass tissue homogeniser (VWR) depending on the size of the pellet.

6.2.1.9 CO difference spectroscopy

All P450s are thought to originate from a single ancestor (CYP51) and thus exhibit similar folding structures (Parvez et al., 2016) and spectroscopic properties. This can be utilised to determine the presence of correctly folded P450 and to quantify the amount of expressed P450 present. To do this, 100 µL of recombinant protein (containing 0.05% BSA), 1.9 mL of potassium phosphate buffer and a pinch of sodium hydrosulfite (Sigma) was added to a 15 mL Falcon tube. The sample was vortexed and left at room temperature for one minute. After one minute the solution was divided into two cuvettes (sample and reference cuvettes) (Hellma Analytics) and placed into a double beam Specord® 200 PLUS spectrophotometer (Analytik Jena). The baseline reference was recorded according to the manufactures protocol. The sample cuvette was removed and carbon monoxide was bubbled through the solution at a rate of 2 bubbles per second for 30 seconds. In this process the reduced (ferrous) form of the protein binds to carbon monoxide, forming a complex. The sample cuvette was placed back into the spectrophotometer and the absorbance was recorded. Correct folding of the recombinant P450 was assessed by the presence of the characteristic peak at 450 nm (see appendix 4 for examples). A greater peak at 420 nm indicates incorrect folding and thus loss of function of the recombinant P450 (Klingenberg, 1958). The amount of P450 in the sample was calculated using the following formula:

P450 amount (nmol/mL)= (absorbance at 450 nm-absorbance at 490 nm)/0.091 X 2000/100

The protein concentration was determined by Bradford as described in section 2.10. All recombinant P450s were snap frozen in liquid nitrogen and stored at -80°C in 500 µL aliquots.

6.2.1.10 Kinetic assays

6.2.1.10.1 Assay Optimisation

It is well known that temperature can effect enzyme activity (Daniel and Danson, 2013). Thus, prior to kinetic assays the optimal assay temperature was determined. As mentioned above, LC-MS analysis can be expensive and time consuming and so assay optimisation was performed using a randomly selected recombinant CYP (CYP9BU2) and a catalytically active P450 model substrate (BFC) as follows:

Temperature optimisation tests were carried out in black flat-bottomed 96-well assay plates (4titude) in a 100 µL reaction containing recombinant P450 (2 pmol), NADPH (1mM) (Sigma) and BFC substrate (50 mM) (Sigma). Samples were incubated at 25, 30, 35 and 40^{o}C for three hours. Three replicates were performed for each data point. Samples incubated without NADPH and wells containing only potassium phosphate buffer served as controls. Data were recorded every minute using a SpectraMax Gemini XPS (Molecular Devices), at the excitation/emission wavelength of 410-510 nm. In order to calculate P450 activity, the fluorescence measurements of controls were subtracted from the fluorescence measurements of samples.

6.2.1.10.2 Insecticide and alkaloid metabolism assays

Microsomal membrane fractions were prepared according to standard procedures (Phillips and Shephard, 2006). Briefly, ~30 snap frozen (-80°C) *O. bicornis* adult females were pulverised in 15 mL of homogenisation buffer using a tissue grinder. The homogenate was centrifuged at 4,000 g for 10 minutes at 4°C. The supernatant was transferred and centrifuged again at 15,000 g for 15 minutes at 4°C. Microsomes were pelleted by further centrifugation at 100,000 g for 1 hour at 4°C. Resulting pellets were resuspended in 1-5 mL of buffer R depending on pellet size. All microsomal membrane fractions were stored at -80°C in 500 µL aliquots.

Michaelis-Menten kinetics for thiacloprid and imidacloprid were performed by

incubating native microsomes (1.6 mg/well) or recombinant P450/CPR (5 pmol/well) with a range of substrate concentrations (200-3.1 µM) in the presence of an NADPH regeneration system at 30±1^{o}C, with shaking, for 1 hour. Three replicates were performed for each data point and the total assay volume was 200 µL. Samples incubated without NADPH and wells containing only potassium phosphate buffer served as controls. The reactions were terminated by the addition of 800 µL of 80% ice-cold acetylnitrile. Samples were then incubated at 4°C overnight and centrifuged at 3,000 g for 10 minutes to pellet any precipitation of protein. LC-MS was carried out in house by Bayer CropScience. Kinetic parameters (K_m and V_{max}) were determined by fitting the Michaelis-Menten equation using Graph Pad Prism v.6 (GraphPad, USA).

In order to explore the metabolism of the insecticides acetamiprid, flupyradifurone, tau-fluvalinate, deltamethrin, coumaphos and chlorpyrifos, and the alkaloids nicotine, anabasine, atropine and hyoscine, native microsomes (1.6 mg/well) or recombinant P450/CPR (5 pmol/well) were incubated with a single concentration of substrate (10 µM) as described above. Two additional samples incubated without NADPH were included in assays where the depletion of the parent compound was assessed (pyrethroids and organophosphates).

All calculations were carried out using Microsoft Excel and Graph Pad Prism v. 6. A paired t-test was used to assess the significance of the depletion of samples incubated with NADPH compared to samples incubated without NADPH.

6.2.1.10.3 P450 model substrate metabolism assays

The compound structure, molecular weight and required fluorescent wavelengths of all of the tested model substrates are shown in tables 6 and 7. Model substrate assays were carried out in black flat-bottomed 96-well assay plates in a 100 µL reaction containing native microsomes (50 µg) or recombinant P450/CPR (2 pmol), NADPH (1 mM) (Sigma) and a P450 model substrate (50 mM) (Sigma). Samples were mixed and incubated at 30^{o}C for 60 minutes. Samples incubated without NADPH and wells containing only

potassium phosphate buffer served as controls. *Musca* microsomes served as an additional comparative control during microsomal assays. Three replicates were performed for each data point. Data were recorded after 60 minutes using a SpectraMax Gemini XPS, at the excitation/emission wavelength suitable for each model substrate. As EC and MC have a similar emission wavelength to NADPH (465 vs. 460 nm respectively), reactions containing these model substrates were terminated prior to measurement by the addition of 100 µL of a stop solution (25% DMSO (Fisher), 0.05 M Tris/HCL pH10 (Fisher), 5 mM glutathione oxidised (Sigma), and 0.2 U glutathione reductase (Sigma)). The reaction was incubated at 30^{o}C for a further 15 minutes and data were recorded at an excitation/emission wavelength suitable for the substrate. 7-hydroxy-4-(trifluoromethyl)-coumarin (HFC) (Sigma) was used to generate a standard curve for model substrates BFC, EFC, MFC, MOBFC, and BOMFC, 7-hydroxycoumarin (HC) (Sigma) for substrates EC and MC, and resorufin sodium salt (Sigma) for substrates BOMR, BR, ER, MR, OOMR, and PR. Each compound was diluted to a range of concentrations (0 (control), 5, 10, 15, 20, 30, 50, 60, 80 and 100 pmol) using potassium phosphate buffer. 100 µL of each concentration was added to each well. The fluorescence was measured using a SpectraMax Gemini XPS, at the excitation/emission wavelength suitable for each compound (see tables 6 and 7). In the case of HC, 100 µL of stop solution was added and mixed prior to fluorescence measurement. Four replicates were performed for each data point. Calculations were carried out using Microsoft Excel. Control measurements were subtracted from substrate measurements and the ‘TREND’ function was used to calculate the y intercept.

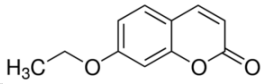
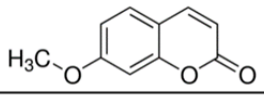
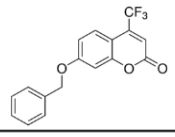
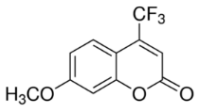
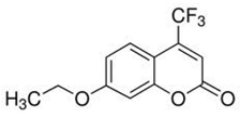
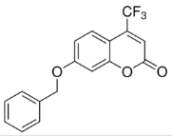
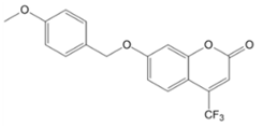
Substrate name	Molecular weight	Chemical structure	Excitation- emission fluorescence wavelength (nm)
7-Ethoxy-coumarin (EC)	190.2		390-465
7-Methoxy-coumarin (MC)	176.17		390-465
7-Benzyloxy-4-(trifluoromethyl)-coumarin (BFC)	320.26		410-535
7-Methoxy-4-(tri-fluoromethyl)-coumarin (MFC)	244		410-535
7-Ethoxy-4-trifluoro-methylcoumarin (EFC)	258.19		410-510
7-(benzyloxymethoxy)-4-trifluoromethylcoumarin (BOMFC)	320.26		405-510
7-(4-methoxybenzyloxy)-4-trifluoromethylcoumarin (MOBFC)	350.29		405-510

Table 7. The molecular weight, chemical structures and excitation- emission wavelengths of P450 fluorescent coumarin based model substrates used during this study.

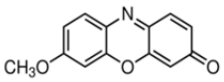
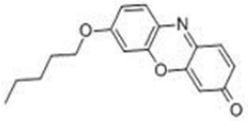
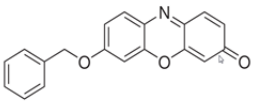
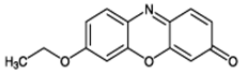
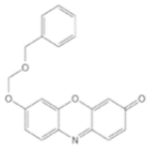
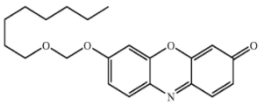
Substrate name	Molecular weight	Chemical structure	Excitation- emission fluorescence wavelength (nm)
Methoxy-resorufin (MR)	227.26		535-590
Pentoxy-resorufin (PR)	283.33		535-590
Benzyloxy-resorufin (BR)	303.11		535-590
Ethoxy-resorufin (ER)	241.2		535-590
(benzyloxymethoxy)resorufin (BOMR)	333.34		520-590
(octyloxymethoxy)resorufin (OOMR)	355.43		520-590

Table 8. The molecular weight, chemical structures and excitation- emission wavelengths of P450 fluorescent resorufin based model substrates used during this study.

6.2.2 Transgenic *Drosophila*

6.2.2.1 Synthesis of candidate genes

Candidate genes were synthesized as described in section 6.2.1.3 but with the following variations. Genes were codon optimised for *D. melanogaster* and *EcoR*I and *Xba*I restriction sites were added up and downstream to the gene sequences respectively. Genes were delivered in GeneArt default vectors encoding kanamycin resistance.

6.2.2.2 Cloning of synthetic genes into the pUAST vector

The synthetic genes were extracted from the GeneArt default vector by carrying out a double restriction digest using the restriction enzymes *EcoR*I and *Xba*I as described in section 2.5. Gel electrophoresis was used to separate and extract the gene from the vector as described in section 2.6. Resulting products were purified as described in section 2.8.1 and cloned into the pUAST vector (see appendix 3 for vector map) that had also been cut with *EcoR*I and *Xba*I as described in section 2.7. Resulting plasmids were purified as detailed in section 2.8.2.

6.2.2.3 Preparation of construct containing three P450 genes (trisophila)

To aid the transcription of each gene, a UAS and a SV40 Poly adenylated (PolyA) were engineered upstream and downstream of each gene sequence depending on its location in the final construct (see figure 28). The UAS acts as a transcriptional promotor, and the PolyA is important for transcription termination (3' polyadenylation). 'Spacers', which consisted of a random genomic DNA sequence of ~1,000 bp from *B. terrestris,* were also included between each gene in order to ensure the termination of transcription of one gene before the initiation of the next. The construction of spacers is detailed in section 6.2.2.4. Restriction sites were also included up- and downstream of the gene sequences.

PCR was used to amplify the PolyA, UAS and the gene from the pUAST vector produced in section 6.2.2.2 as detailed in section 2.4.2. PCR reactions were carried out as described in section 2.4.2 and contained Phusion® High-fidelity DNA polymerase and primers encoding restriction sites (see appendix 2 for primer sequences). The band of the correct size was then extracted from agarose gel and purified as described in section 2.8.1.

Cloning of PCR products straight into pUAST was found to be very inefficient, and thus PCR products were subcloned into the pJET 1.2 vector (see appendix 3 for vector map) as described in section 2.7. The plasmids were then sent for sequencing (see appendix 2 for primer sequences) as described in section 2.9

in order to confirm that the UAS and PolyA had been successfully amplified from the pUAST vector.

Once confirmed, each gene was cut out of the pJET vector using the respective restriction enzymes (see figure 27). *Cyp9bu2* was found to be too similar in size to the pJET vector to effectively gel extract (2,635 bp vs. 2,651 bp respectively) and so *Bg*II was used to cut the vector into two smaller fragments (1,786 and 865 bp), allowing for the separation of *cyp9bu2*.

All three genes were then cloned into the pUAST vector that was previously cut with *EcoR*I and *Xba*I using MAXefficiency DH5α competent cells as detailed in section 2.7. Successful insertion was confirmed by colony PCR and by a diagnostic restriction digest as described in section 2.5.

The resulting construct along with the pJET vector containing spacer 1 were cut with *Kpn*I and purified as described in section 2.5 and section 2.8.2 respectively. The products were run on a gel as described in section 2.6 to check for any unspecific cutting. Spacer 1 was cloned into the pUAST vector containing the three genes as described in section 2.7. This process was then repeated in order to insert spacer 2 into the construct. Ligating spacer 2 into the construct proved problematic and so shrimp alkaline phosphatase (rSAP) was used according to the manufacturers protocol to remove 5' and 3' phosphates from the vector, preventing re-ligation of the linearised vector. The final construct was then sequenced as detailed in section 2.9 to confirm the insertion of all three genes and the two spacers.

6.2.2.4 Construction of spacer 1 and spacer 2

Spacers were amplified from genomic DNA extracted from two *B. terrestris* adult workers using the E.Z.N.A® insect DNA kit (Omega bio-tek) following the manufacturers protocol. The quality and quantity of extracted DNA was assessed using a NanoDrop1000 spectrophotometer and gel electrophoresis as detailed in section 2.2. Phusion® High-fidelity DNA polymerase was used to amplify DNA sequences following the manufactures protocol using sequence-

specific primers containing selected restriction sites (see figure 27). PCR products were purified as described in section 2.8.2. Each spacer was individually cloned into the pJET vector as detailed in section 6.2.2.3.

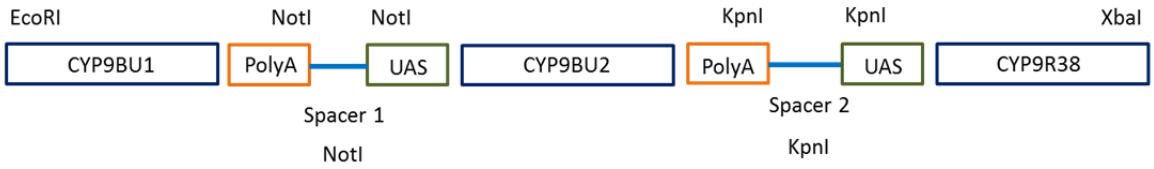


Figure 27. Order of genes and spacers in the pUAST vector along with the restriction site locations.

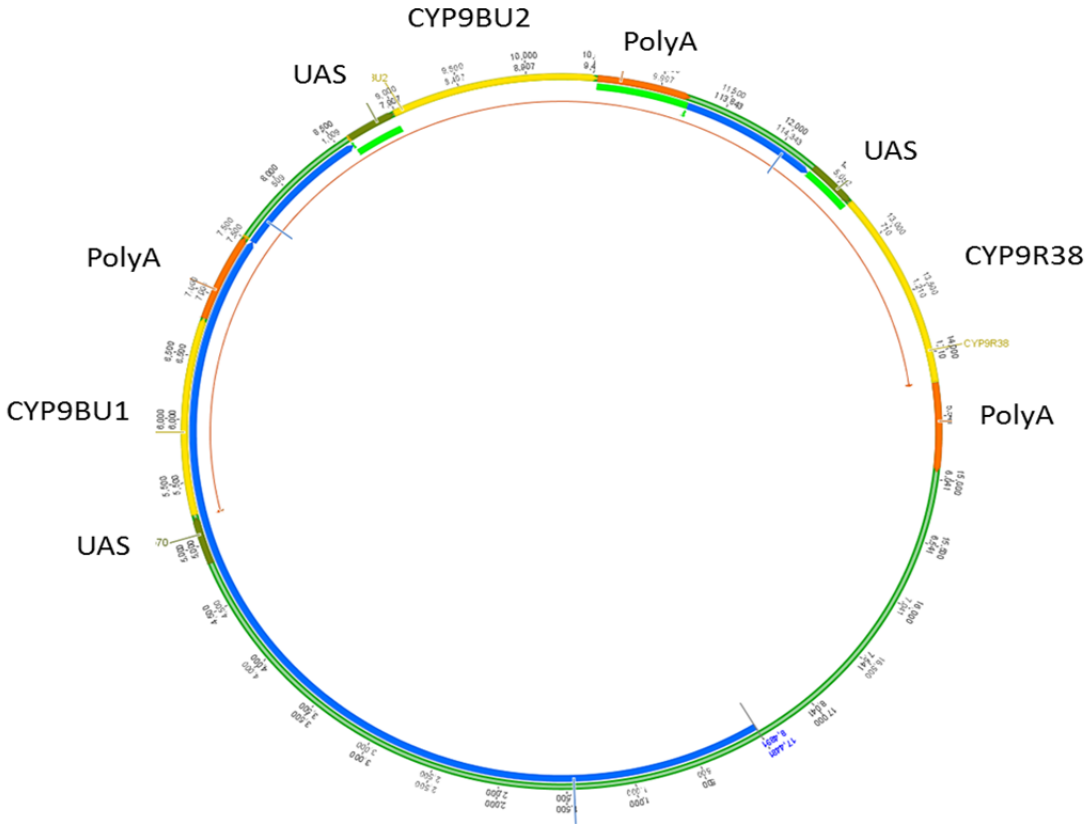


Figure 28. Diagram of the final construct (17.5kb) microinjected into *Drosophila* embryos for the production of the trisophila line. Image was produced using Geneious.

6.2.2.5 Microinjection of plasmids into *Drosophila* embryos

~400 ng of vectors were sent to the fly facility at the University of Cambridge for microinjection into *Drosophila* embryos and the creation of balanced lines. Briefly, using the PhiC31 system, clones were transformed into the germ line of strain 13-20 attP40 carrying the attP docking site on chromosome 2 ["y1w67c23; P attP", 1;2"] with balanced lines.

6.2.2.6 GAL4/UAS targeted gene expression

Virgin females of the Act5C-GAL4 strain, heterozygous for GAL4 (under the control of actin) with the phenotype of curly wings and white eyes, were collected over a period of 5 days. 15 virgin females were then placed in a 250 mL vial containing fly food (see appendix 1 for recipe) with 12 males homozygous for the transgene/UAS promotor as identified by the phenotype of red eyes and straight wings (see figure 29). After seven days the adult flies were removed and the development of the progeny was monitored over 2 weeks. The resulting female F1 progeny displaying the red eye straight wing phenotype were used in subsequent insecticide bioassays.

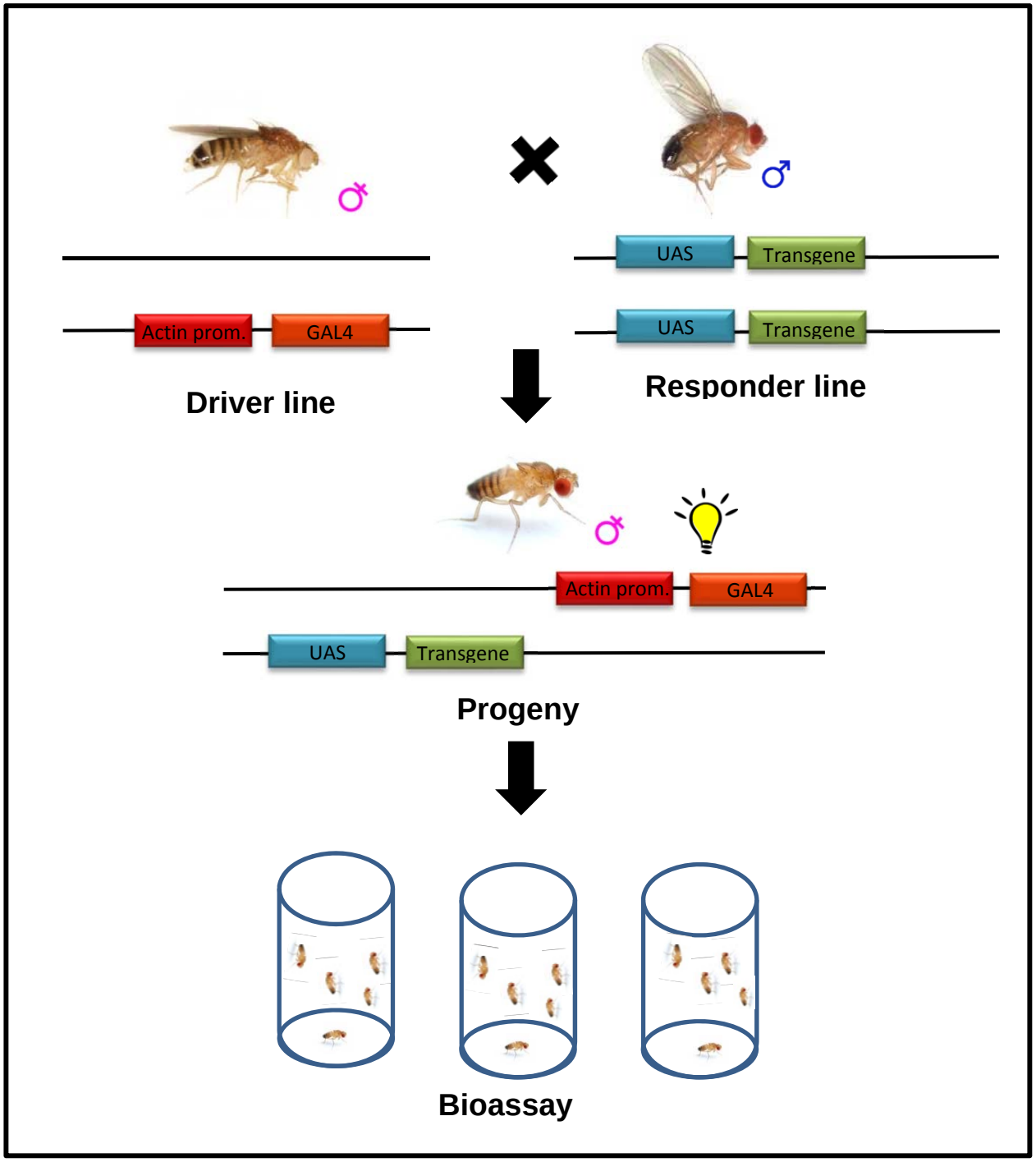


Figure 29. The GAL4/UAS targeted gene expression system, consisting of a driver line containing GAL4 and a promoter (female line), and a responder line containing an upstream activating sequence (UAS) and the gene of interest (male line). Upon crossing of these lines, GAL4 is produced and binds to the UAS, activating the transcription and expression of the transgene in the progeny. Female progeny are used in subsequent insecticides bioassays.

6.2.2.7 Verification of transgene expression in *D. melanogaster*

Expression of transgenes in transgenic *D. melanogaster* lines were confirmed by qPCR using gene-specific primers (see appendix 2) as described in section 2.4.3. Relative quantification was carried out between adult females of the parent line displaying the phenotype red eye straight wings compared to adult females of the F1 progeny displaying the same phenotype. The assay consisted of two technical replicates and four biological replicates consisting of five flies. Transgenes were considered overexpressed when the resulting Ct values of the F1 progeny were greater than that of the parental line.

6.2.2.8 Insecticide bioassays

Sugar-agar vials were prepared for insecticide bioassays as follows. 5 g of agar (Sigma) and 3 g sugar (Sigma) were added to 250 mL of boiling tap water, stirring slowly until the sugar had dissolved. The solution was left to cool to 70°C and 1 mL of acetic acid (Sigma) was added. 2 mL of the resultant sugar-agar solution was then transferred into 95 x 25 mm vials and allowed to set. Once set, 100 µL of the corresponding concentration of imidacloprid, thiacloprid or acetamiprid (see table 9) was added to each vial. Dilutions were carried out using an acetone:water solution (1:1). In the case of the controls, 100 µL of acetone:water (1:1) was applied. The vials were gently vortexed to ensure the even spread of the solution and placed in a fume hood overnight to allow the solution to evaporate. Bioassays were started by the addition of 20 adult females of the F1 progeny displaying the red eye straight winged phenotype into each vial. Four replicates were carried out for each concentration. Flies with the same genetic background minus the transgene were used as a control. The mortality of tested flies was assessed after 24 and 48 hours. Probit analysis was carried out to generate LD_{50} values as described in section 3.2.4. Resistance ratios were calculated by dividing the LD_{50} value of the transgenic line by the LD_{50} value of the control line.

Compound	Concentrations (ppm)
Thiacloprid	12,500, 2,500, 1,000, 500, 100, 20, 0
Imidacloprid	2,500, 1,000, 500, 100, 50, 20, 0
Acetamiprid	2,500, 500, 100, 20, 10, 4, 0

Table 9. Concentrations of insecticides used in *D. melanogaster* bioassays to produce dose response curve and LD_{50} values.

6.3 Results

6.3.1 Enzyme kinetic assays

6.3.1.1 Assay optimisation

Incubation of CYP9BU2 with BFC at a range of temperatures revealed that incubation at 30°C resulted in the greatest enzyme activity. Enzyme activity substantially decreased when incubation temperatures were greater than 30°C, likely due to denaturation of the enzymes. The reaction rate was still within the linear range after 60 minutes incubation and so this was used as the incubation time in future assays (figure 30).

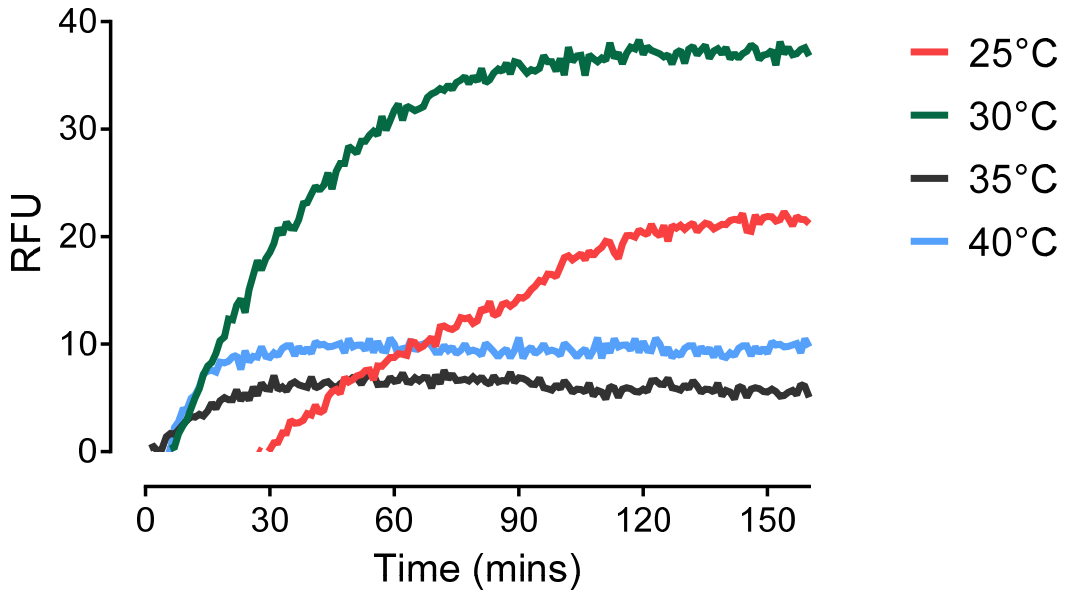


Figure 30. CYP9BU2 enzyme activity (fluorescence) against the P450 model substrate BFC incubated at 25, 30, 35, and 40°C over a 160-minute period. Data points are mean values (n=3).

6.3.1.2 Insecticide metabolism assays

Female *O. bicornis* microsomes were found to be capable of metabolising both neonicotinoids into their hydroxylated forms (5-hydroxy thiacloprid and 5-hydroxy imidacloprid), however substantial variation was seen in their ability to do so. The apparent K_m value for 5-hydroxy thiacloprid was 22.5 µM with an apparent V_{max} of 1.047 mg/mL/min whilst the apparent K_m value for 5-hydroxy imidacloprid was 475 µM with an apparent V_{max} of 2.258 mg/mL/min (figure 31).

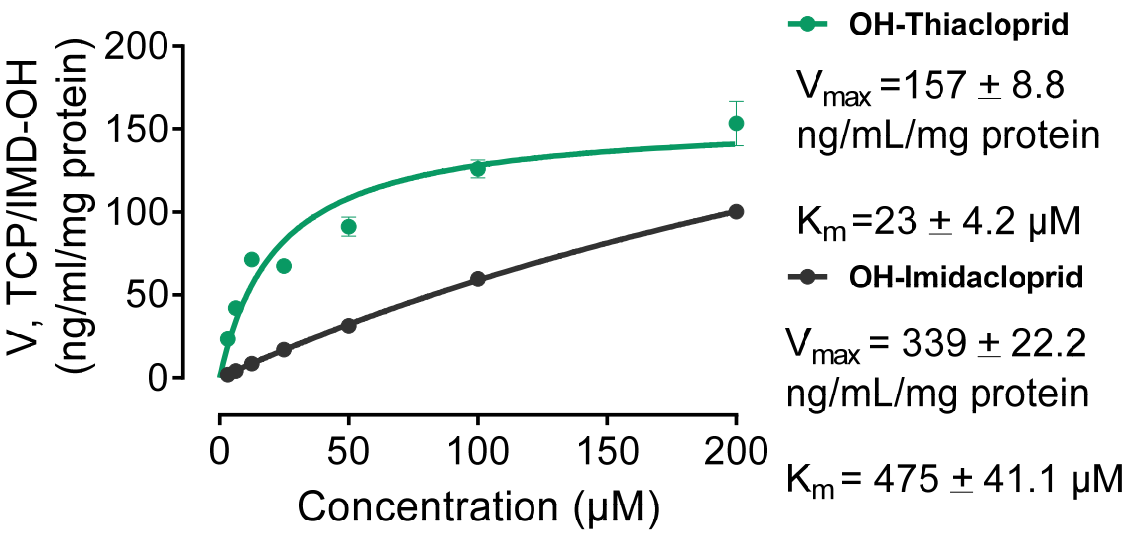


Figure 31. Michaelis-Menten kinetics of imidacloprid and thiacloprid hydroxylation by native *O. bicornis* microsomes. Analysed by non-linear regression. Data points are mean values (n=3 ± SD).

With the exception of CYP336L1, correct folding of recombinant P450s was achieved for all expressed candidate P450s (see appendix 4 for CO difference spectra). Incubations of P450s with thiacloprid followed typical Michaelis-Menten kinetics, producing classical hyperbolic curves. In brief, at low substrate concentrations, the reaction rate increased linearly with substrate concentration until enzyme saturation occurred and substrate concentration was no longer the limiting factor of the reaction rate. At this point, the maximum velocity was reached and the rate plateaued (figure 31). Incubations of P450s with imidacloprid did not follow Michaelis-Menten kinetics, producing biphasic graphs with extremely linear results. Due to the atypical Michaelis-Menten kinetics of CYP9R1 and CYP9R38, it was not possible to calculate V_{max} and K_m parameters, which in itself indicates the poor ability of these enzymes to break down this insecticide. Initial incubations of CYP9R39, CYP6AS151, CYP336A36, CYP336A35, and CYP6AS127 with imidacloprid and thiacloprid (10 µM), did not result in any observable hydroxylation and thus Michaelis-Menten kinetics was not carried out for these P450s.

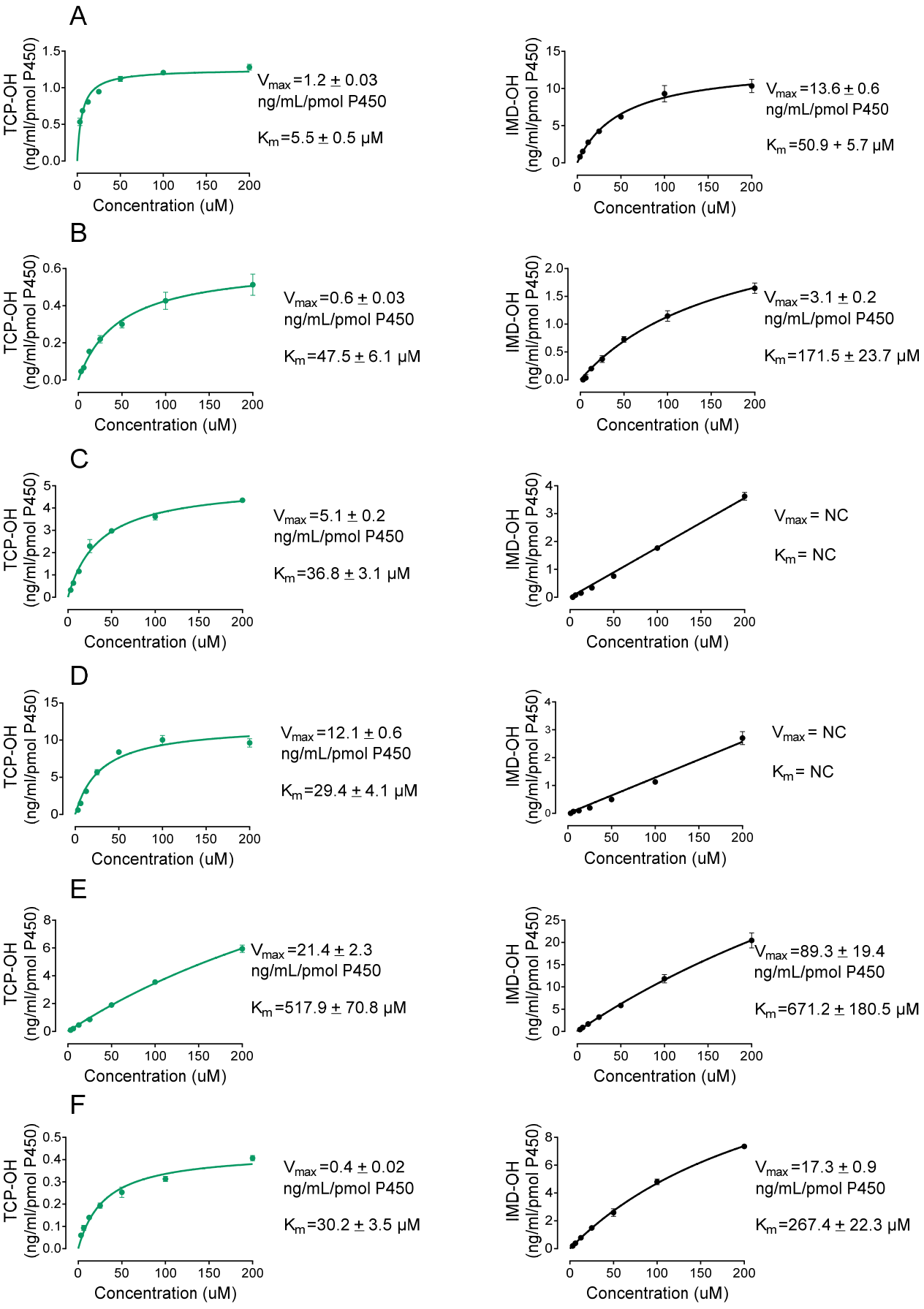


Figure 32. Michaelis-Menten kinetics of thiacloprid (green) and imidacloprid (black) hydroxylation by recombinant P450s CYP9BU1 (A), CYP9BU2 (B), CYP9R1 (C), CYP9R38 (D), CYP9DN1 (E), and CYP6AQ55 (F). Analysed by non-linear regression. Data points are mean values (n=3 ± SD).

Acetamiprid was readily biotransformed into *N*-desmethyl acetamiprid by five of the recombinant P450s tested (predominantly by CYP9BU1 and to a lesser extent CYP9BU2, CYP9R38, CYP9DN1 and CYPAQ55). Similarly, CYP9BU1 exhibited the greatest metabolism of flupyradifurone, followed by CYP9BU2, CYP9R38, CYP9DN1 and CYP9R1.

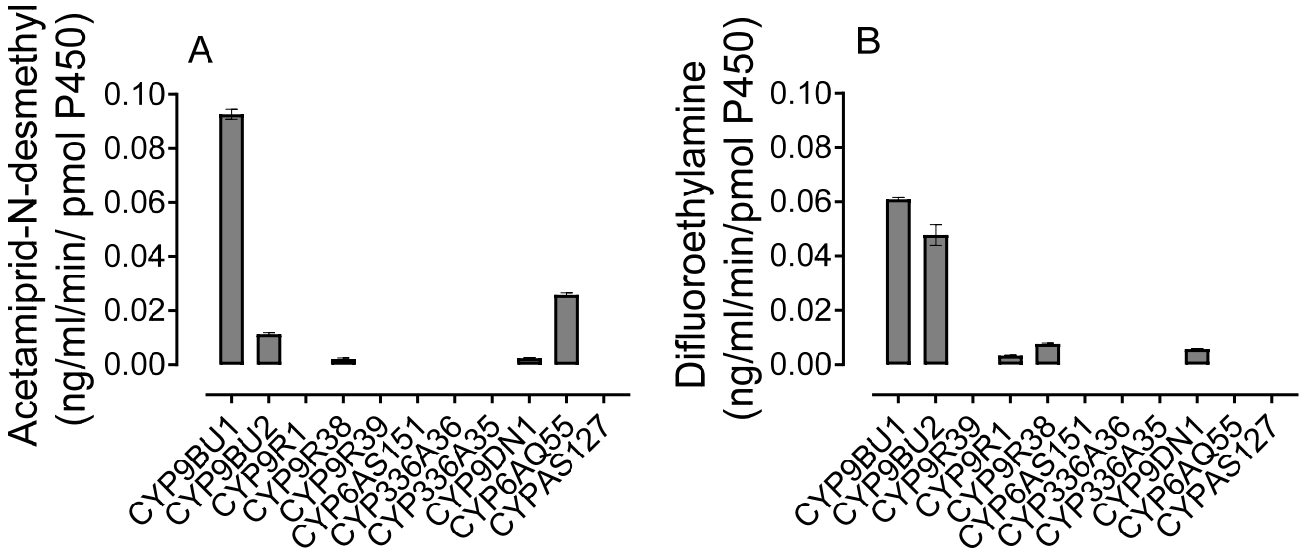


Figure 33. Biotransformation of acetamiprid (A) and flupyradifurone (B) by CYP9BU1, CYP9BU2, CYP9R1, CYP9R38, CYP9R39, CYP6AS151, CYP336A35, CYP9DN1, CYP6AQ55, and CYPAS127. Data points are mean values (n=3 $\pm$ SD).

No significant depletion of the pyrethroids tau-fluvalinate and deltamethrin was observed by recombinant P450s. Although, low levels of tau-fluvalinate depletion was seen by CYP6AS151, CYP9DN1, and CYP6AQ55 and low levels of deltamethrin depletion by CYP336A36, CYP336A35, CYP9DN1, CYPAS127, and CYP9R38.

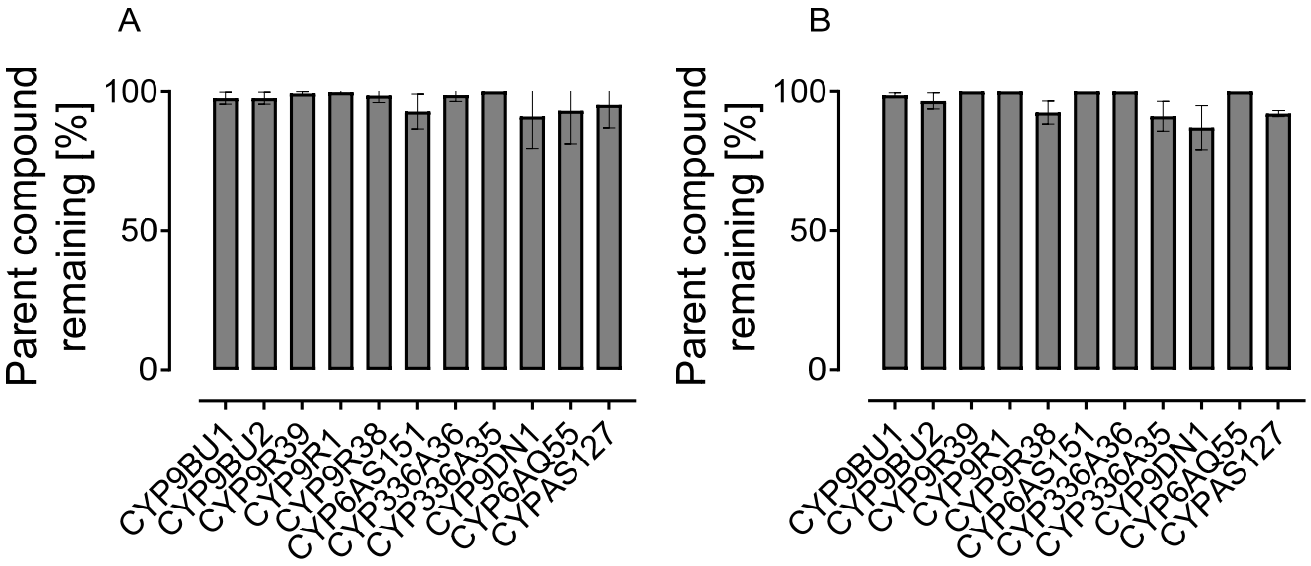


Figure 34. Depletion of tau-fluvalinate (A) and deltamethrin (B) by CYP9BU1, CYP9BU2, CYP9R1, CYP9R38, CYP9R39, CYP6AS151, CYP336A35, CYP9DN1, CYP6AQ55, and CYPAS127. Data points are mean values (n=3 ± SD).

Significant depletion of the organophosphate coumaphos was observed most prominently by CYP336A36 followed by CYP9BU2, CYP336A35 and CYPAS127. Additionally, significant depletion of chlorpyrifos was observed by CYP9R1.

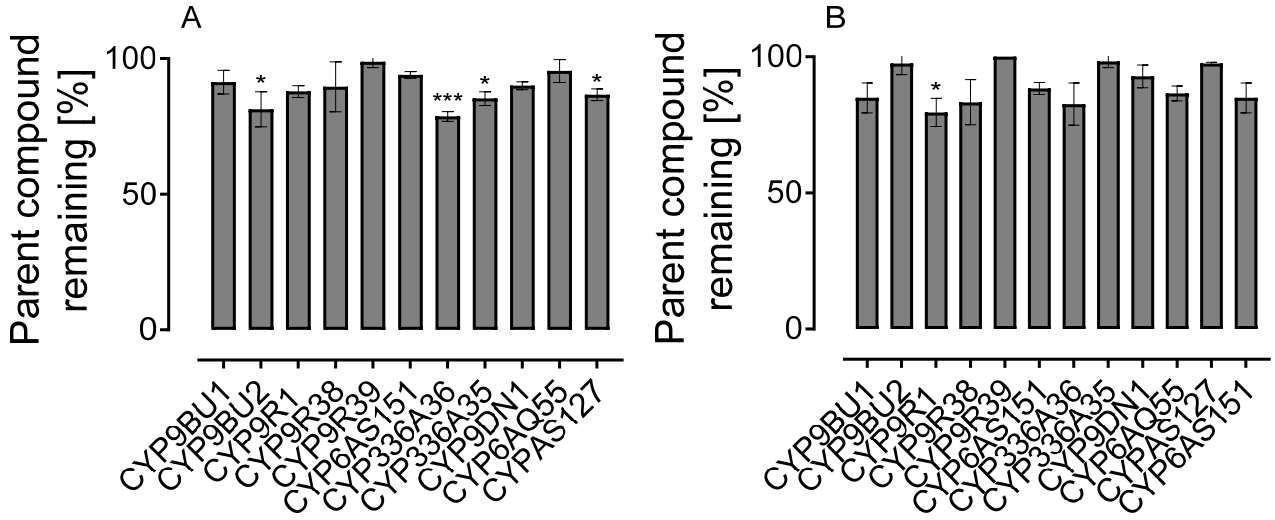


Figure 35. Depletion of coumaphos (A) and chlorpyrifos (B) by CYP9BU1, CYP9BU2, CYP9R1, CYP9R38, CYP9R39, CYP6AS151, CYP336A35, CYP9DN1, CYP6AQ55, and CYPAS127. Data points are mean values (n=3 ±

SD). Significant differences of test samples (NADPH+) vs controls (NADPH-) are denoted by * $p<0.05$, ** $p<0.005$, *** $p<0.0005$; paired t-test).

6.3.1.3 Alkaloid metabolism assays

Significant metabolism of nicotine was observed by microsomes with ~5% of the starting compound remaining after 60 minutes incubation ($p<0.0005$). In the case of recombinant P450s, the greatest depletion of nicotine was displayed by CYP6AQ55, with ~10% of the initial parent compound remaining. Some additional depletion was observed by CYP336A36, CYP6AS127, CYP6AS151 and CYP9DN1 although this was not significant. High levels of depletion of anabasine by microsomes (~40% remaining) was observed, however none of the tested P450s exhibited significant depletion of anabasine. Some depletion of atropine by microsomes was observed (-80% remaining), as well as high levels of depletion by CYP336A35 (~40% remaining). Hyoscine was not significantly depleted by microsomes but some depletion was observed by CYP336A36, followed by CYP9BU2, and CYP9R38, CYP9DN1, CYP9R1 and CYP9BU1 (figure 36).

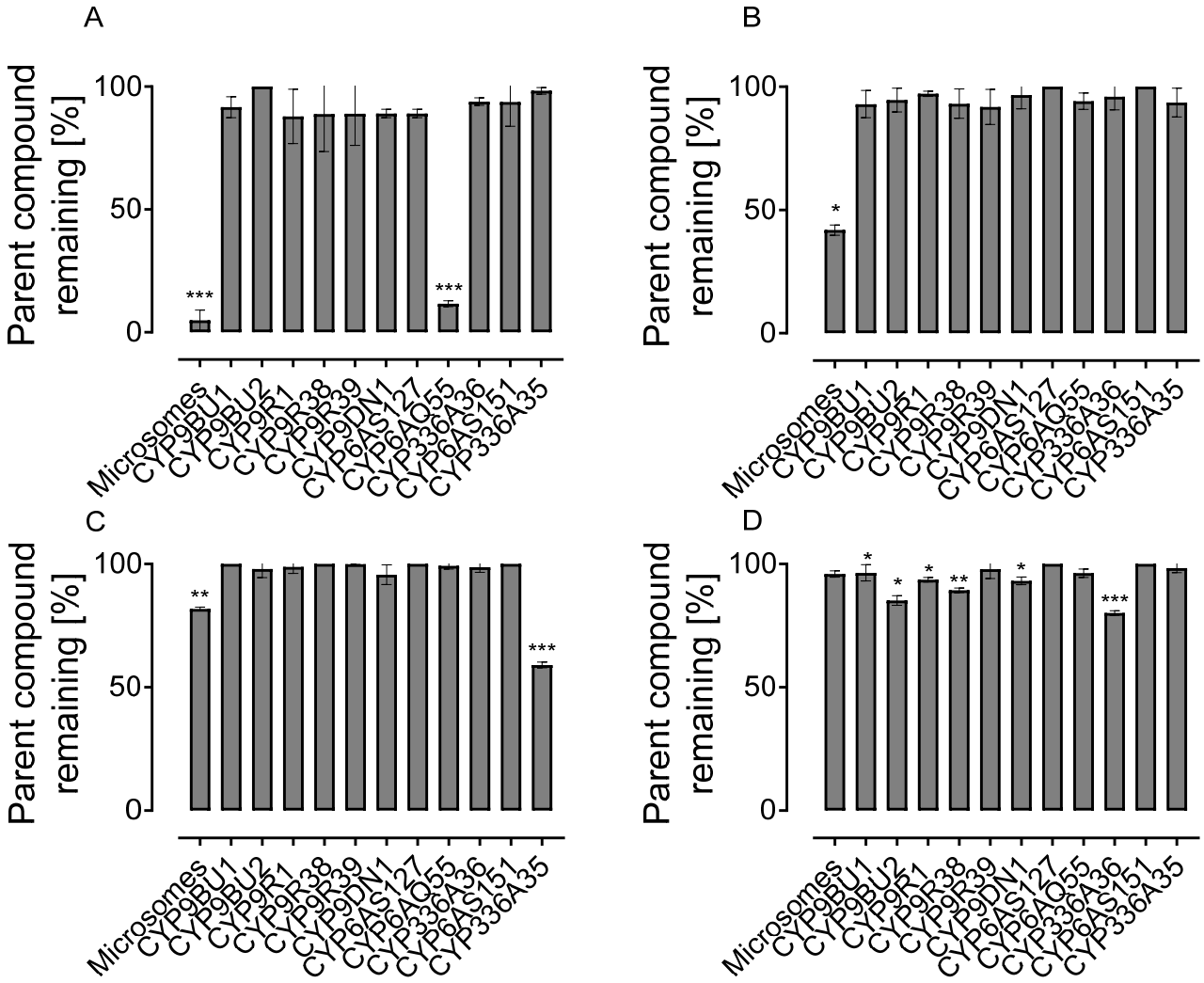


Figure 36. Depletion of 10 µM of nicotine (A), anabasine (B), atropine (C), and hyoscine (D) by female *O. bicornis* microsomes and recombinant P450s after a 60 minute incubation at 30°C. Data points are mean values (± SD). Significant differences of test samples (NADPH+) vs controls (NADPH-) are denoted by * $p<0.05$, ** $p<0.005$, *** $p<0.0005$; paired t-test).

6.3.1.4 P450 model substrate assays

P450 model substrate assays revealed that microsomes extracted from both female and male *O. bicornis* exhibit a greater specific activity for coumarin-derived substrates compared to resorufin-derived substrates, with the highest rate of catalytic activity displayed for EFC and the lowest for BOMFC (figure 38). The catalytic profiles of individual P450s were similar, displaying activity for a broad range of coumarin based substrates but no activity for resorufin based

substrates. Greatest activity was observed for triflouromethyl coumarins such as MOBFC which have greater molecular weight. CYP9R39, an enzyme that did not show any observable hydroxylation of imidacloprid or thiacloprid, displayed lower levels of activity compared to other P450s belonging to the CYP9 family (figure 38).

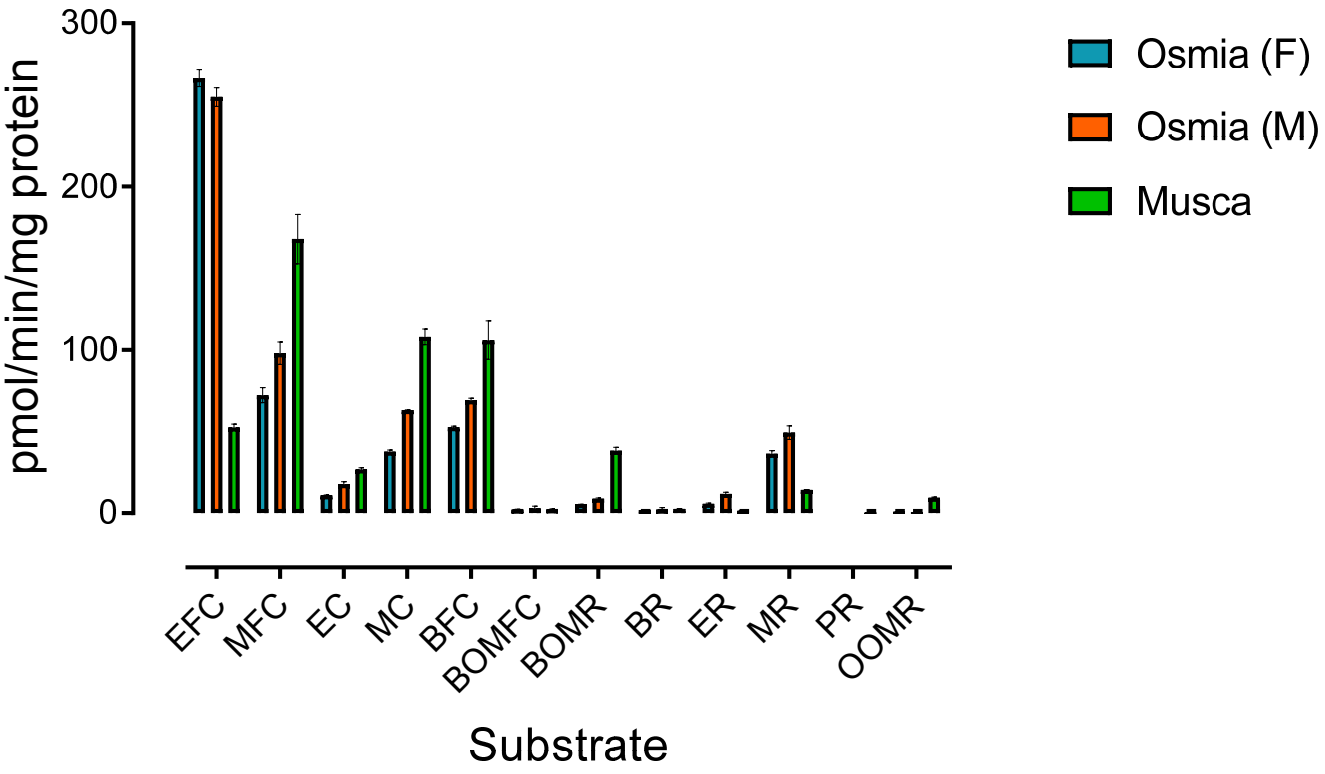


Figure 37. Fluorescence activity of male (M) and female (F) *O. bicornis* microsomes and *M. domestica* microsomes against coumarin- and resorufin-based P450 model substrates. Data points are mean values (n=3 ±SD).

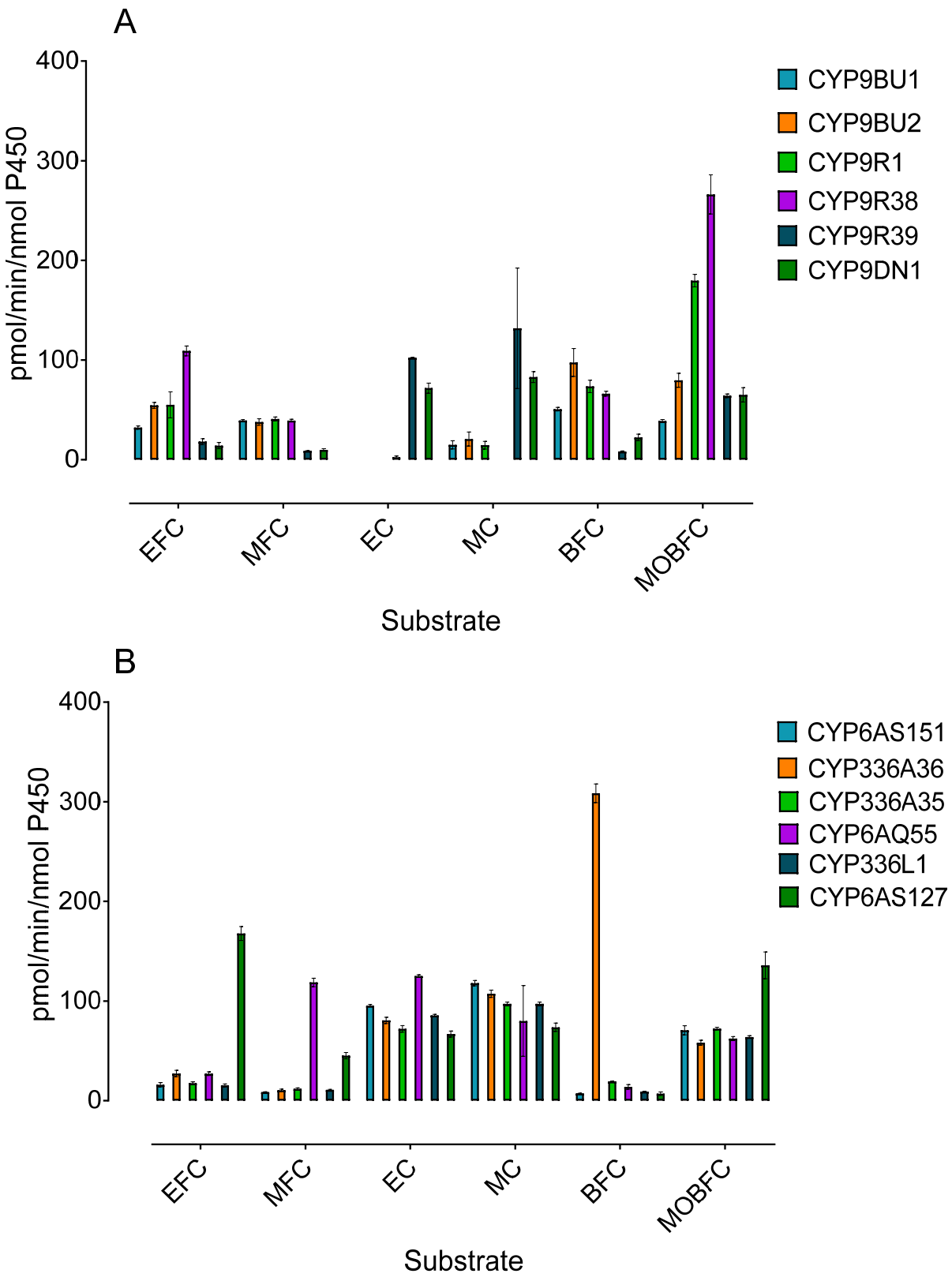


Figure 38. Fluorescence activity of P450s belonging to CYP9 (A) and CYP6 (B) families against coumarin-based P450 model substrates. Data points are mean values (n=3 ±SD).

6.3.2 Transgenic *Drosophila*

Transgenic *Drosophila* expressing CYP9BU1 were found to be significantly resistant to thiacloprid, but not imidacloprid, compared to the control line. *Drosophila* lines expressing CYP9BU2, CYP9R38 and CYP9R39 displayed no significant difference in sensitivity to both compounds compared to the control line. CYP9R1 was found to have detrimental effects to the reproductive output of flies and so the sensitivity of the F1 progeny could not be assessed. Transgenic *Drosophila* expressing CYP9BU1, CYP9BU2 and CYP9R38 in combination (trisophila) displayed significant resistance to thiacloprid, but not imidacloprid, compared to the control line (figure 39).

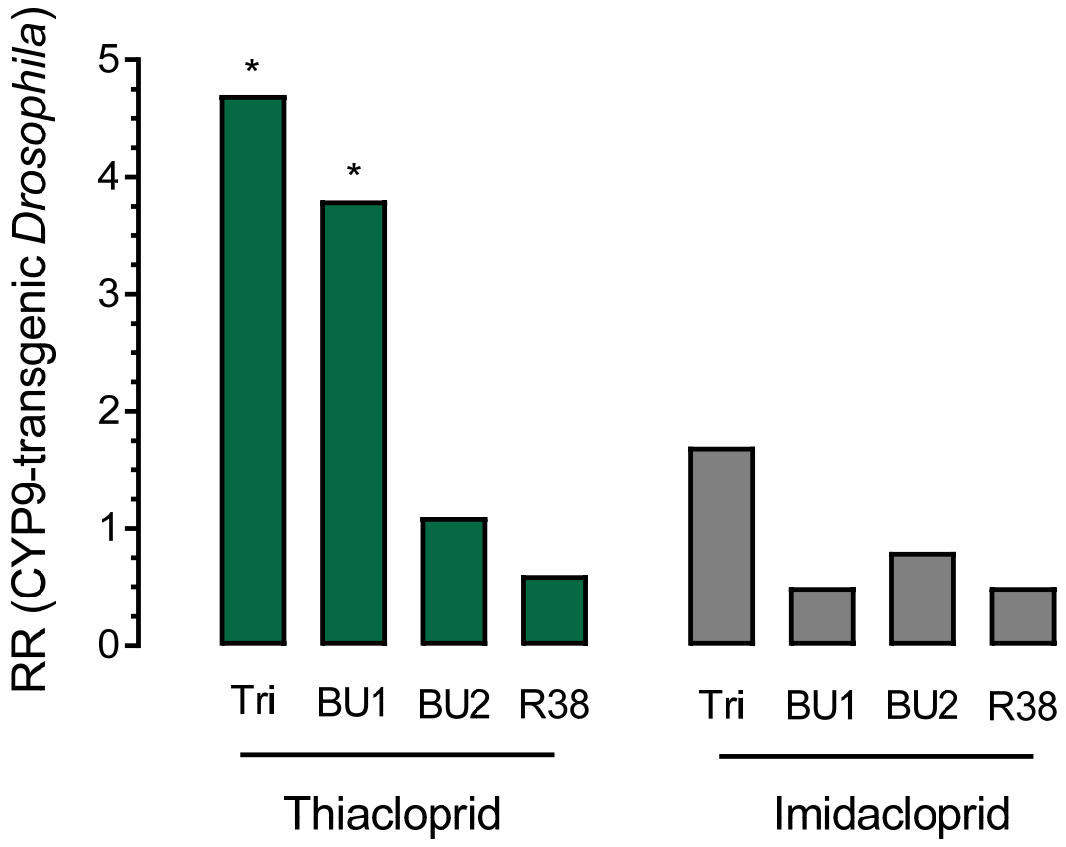


Figure 39. Resistance ratios (RRs) of transgenic *Drosophila* lines expressing CYP9BU1, CYP9BU2, and CYP9R38 in combination (tri) or alone to thiacloprid and imidacloprid compared to a control line (*Drosophila* of the same genetic background minus the transgene). Significance (*) is referenced against the control line based on non-overlapping 95% confidence limits of LD_{50} values.

Genotype	LD_{50} (ppm)	95% CI	Slope	± SE	Resistance ratio
Control	17.03	8.4-27.9	1.2	0.14	1
CYP9BU1	65.4	31.2-111.4	1.1	0.12	3.8
CYP9BU2	17.5	4.9-37.2	1.2	0.13	1.1
CYP9R38	9.8	1.7-26.2	1.4	0.11	0.6
Trisophila	80.5	33.1-151.8	1.3	0.11	4.7

Table 10. LD_{50} values (± 95% confidence intervals), slope (± SE) and resistance ratios of transgenic *Drosophila* lines 48 hours after thiacloprid application.

Genotype	LD_{50} (ppm)	95% CI	Slope	± SE	Resistance ratio
Control	165.03	131-207.1	1.7	0.12	1
CYP9BU1	85.9	61.9-115.2	1.3	0.13	0.5
CYP9BU2	135.4	97.2-185.5	1.5	0.14	0.8
CYP9R38	79.1	50.1-117.6	1.6	0.15	0.5
Trisophila	285.2	163.7-419.2	1.5	0.21	1.7

Table 11. LD_{50} values (± 95% confidence intervals), slope (± SE) and resistance ratio of transgenic *Drosophila* lines 48 hours after imidacloprid application.

6.4 Discussion

6.4.1 Metabolic capabilities of microsomes and recombinant P450s

6.4.1.1 Insecticides

Initially, the overall P450 activity of *O. bicornis* was assessed by measuring the biotransformation of neonicotinoids into their hydroxylated forms by native

microsomes. These microsomal proteins were found to exhibit a greater binding affinity to thiacloprid compared to imidacloprid, as demonstrated by the considerably lower apparent K_m value generated from Michaelis-Menten kinetics. This result is suggestive of a greater capability to metabolise thiacloprid. It must be noted that Michaelis-Menten kinetics was originally designed for reactions involving a single substrate binding site (Atkins, 2005). As microsomal preparations contain multiple P450 enzymes and thus multiple binding sites, the kinetic parameters generated may not be strictly accurate but still allow for an initial comparison of the metabolism of the two insecticide substrates.

To further explore the neonicotinoid detoxification capabilities of *O. bicornis*, the specific P450s involved in their metabolism were identified by incubating select recombinant P450s belonging to the CYP3 clade with either imidacloprid or thiacloprid and analysing the resultant metabolites using LC-MS. Of the 11 successfully expressed P450s, six were able to metabolise both imidacloprid and thiacloprid, five of which belong to the CYP9 family, and one to the CYP6 family. Similar to microsomal preparations, all catalytically active P450s displayed a greater affinity for thiacloprid compared to imidacloprid, as demonstrated by the lower K_m values. In particular, CYP9BU1, which is the most closely related orthologue of *A. mellifera* CYP9Q3 (49% identity), exhibited the greatest binding affinity for thiacloprid. The superior affinity of these P450s for thiacloprid could enable its rapid metabolism before any detrimental effects can occur, explaining, at least in part, its comparatively low toxicity.

To further characterise these P450s, the metabolism of acetamiprid, a second cyano-substituted neonicotinoid that exhibits low toxicity to *O. bicornis*, was assessed. Acetamiprid was readily biotransformed into *N*-desmethyl acetamiprid, predominantly by three recombinant P450s (CYP9BU1 and to a lesser extent CYP9BU2, and CYPAQ55). The ability of these P450s to metabolise acetamiprid was far greater than that of imidacloprid, providing further evidence for the superior ability of *O. bicornis* to metabolise cyano- compared to nitro-substituted neonicotinoids, reflecting their substantially lower toxicity. Similarly, the butenolide flupyradifurone, which also exhibits low topical toxicity to *O. bicornis* (LD_{50} >100 µg/bee), was found to be predominantly

metabolised by CYP9BU1.

Performing LC-MS analysis requires the definitive identification of metabolites and the preparation of synthetic standards (Obach and Reed-Hagen, 2002). Synthetic standards were not available for the pyrethroids (tau-fluvalinate and deltamethrin) or the organophosphates (coumaphos and chlorpyrifos) tested and thus the formation of specific metabolites could not be measured. Instead, P450 metabolism was inferred from the depletion of parent compounds, specifically by comparing the concentration of parent compounds in reactions with and without NADPH. It is noted that this method may not have been as accurate as measuring metabolite production, especially for detecting low levels of metabolism. Synergist bioassays detailed in chapter 3 revealed significant synergism when PBO was co-applied with tau-fluvalinate and to a lesser extent deltamethrin, suggesting the involvement of P450s in the metabolism of pyrethroids. However, no significant depletion of either deltamethrin or tau-fluvalinate was observed by the P450s expressed in this chapter, suggesting alternative P450s, or alternative detoxification enzymes may play a role in their metabolism. A study by Johnson et al. (2006) found that co-application of a CCE inhibitor, S,S,S-tributyl phosphorotrithioate (DEF), with tau-fluvalinate on *A. mellifera* resulted in low levels of synergism, suggestive of the involvement of CCEs in tau-fluvalinate metabolism, albeit to a lesser extent. It is possible that CCEs or indeed other P450s not characterised in this study may also be contributing to tau-fluvalinate metabolism by *O. bicornis*. As for pyrethroids, the metabolic capabilities of the organophosphates coumaphos and chlorpyrifos were assessed by the depletion of parent compounds. Four of the tested P450s exhibited significant levels of depletion of coumaphos (CYP9BU2, CYP336A35, CYP9DN1, and CYPAS127) whereas only one P450 displayed significant depletion of chlorpyrifos (CYP9R1), suggesting that the greater tolerance of *O. bicornis* to coumaphos compared to chlorpyrifos may be due to a greater detoxification ability by P450s.

P450 model substrate profiles of both whole microsomes and recombinant P450s revealed a substantial preference for coumarin-derived substrates compared to resorufin-derived substrates. Slight variation was observed between the specific activity of female and male microsomes, with males

exhibiting slightly higher activity against almost all of the tested substrates. This is likely due to the presence of the venom sac in the female microsomes, which has previously been found to contain P450 inhibitors that are released during homogenisation (Gilbert and Wilkinson, 1975) (although initial tests of microsomes prepared with and without the venom sac showed little variation in P450 activity). All of the recombinant P450s tested displayed broad substrate specificity, showing functional activity for almost all of the coumarin based substrates tested. This broad specificity is suggestive of a promiscuous active site, which could explain how these P450s are able to metabolise a number of different insecticides.

Exposure of *O. bicornis* to multiple insecticides that are metabolised by these P450s could lead to substantial synergism due to competition between compounds for the active site. Johnson et al. (2009) explored the synergistic interactions of coumaphos and tau-fluvalinate, which can reside in the wax of honey bee hives for years after *Varroa* treatments. The toxicity of coumaphos was found to increase with increasing tau-fluvalinate concentrations and vice versa. Both of these compounds are able to fit into the same catalytic pocket of CYP9Q P450s, which could explain the observed synergism (Mao et al., 2011).

6.4.1.2 Alkaloids

O. bicornis microsomes displayed moderate depletion of anabasine, however none of the tested P450s exhibited significant metabolism of this compound, suggesting that other P450(s) not tested in this study are involved in the metabolism of anabasine seen by microsomes. Significant metabolism of atropine by microsomes was observed and is likely due to metabolism by CYP336A35. Even though no significant metabolism of hyoscine was observed by native microsomes, low-level metabolism was observed by a number of P450s (in particular CYP336A36), suggestive of some involvement of P450s in the metabolism of this compound. As these alkaloids are similar in chemical structure (see section 1.4.4), it is plausible that these P450s are able to metabolise a number of these compounds.

Incubation of native microsomes with nicotine revealed significant depletion of

the parent compound, demonstrating that P450 enzymes are able to efficiently break down nicotine. Further studies with recombinant P450s identified CYP6AQ55 as a major metaboliser of nicotine. Only a few other insect species are known to tolerate nicotine in their diet (du Rand et al., 2017b), and the ability of this P450 to metabolise nicotine rivals that of insect pest species e.g. 60-minute incubation of CYP6CY3, implemented in the nicotine resistance of *M. persicae nicotianea,* with a 10 µM nicotine solution resulted in 90% depletion (Bass et al., 2013).

As neonicotinoid insecticides are based on the chemical structure of nicotine it was expected that cross-resistance between nicotine and neonicotinoids would occur. Members of the CYP9 family involved in neonicotinoid metabolism exhibited low levels of metabolism of nicotine, and similarly CYP6AQ55 was able to metabolise thiacloprid, imidacloprid and acetamiprid, albeit to a lower degree than members of the CYP9 family, suggesting cross-resistance may be occurring. Taken together, these results suggest that *O. bicornis* may have evolved a natural detoxification mechanism that allows tolerance of low levels of SPMs in nectar and pollen. Additionally *O. bicornis* can utilise this mechanism to detoxify the similarly structured synthetic neonicotinoids. Indeed, this ability to metabolise nicotine is not restricted to *O. bicornis* but also extends to other bee species such as *A. mellifera*, providing further evidence for an evolved metabolic mechanism in bees (du Rand et al., 2017b), although further work is needed to strengthen this conclusion. As a further study, it would be interesting to determine the SPM detoxification capabilities of other important pollinators such as lepidopterans in order to see whether they have also evolved similar mechanisms in response to SPM exposure.

6.4.2 Transgenic *Drosophila*

Acute toxicity bioassays of transgenic *Drosophila* lines revealed that the expression of CYP9BU1 alone is sufficient to confer resistance to thiacloprid but not to imidacloprid. These results further validate the key role of CYP9BU1 in the metabolism of thiacloprid. In contrast to kinetic studies detailed in section 6.2, no evidence of thiacloprid resistance was observed for lines containing CYP9BU2 and CYP9R38, although kinetic studies only displayed low levels of

metabolism which may have been difficult to detect in the less sensitive *Drosophila* system. Similar to this study, Manjon et al. (2018) found that transgenic *Drosophila* expressing the key thiacloprid-detoxifier CYP9Q3 from *A. mellifera* conferred significant resistance to thiacloprid but not imidacloprid. However, the level of CYP9BU1 resistance seen in this study was lower (~4-fold) than that of CYP9Q3 (>10-fold) suggesting that additional P450s may contribute to thiacloprid metabolism.

The accumulative metabolism of multiple P450 was explored by producing *Drosophila* lines expressing three thiacloprid-detoxifying P450s (CYP9BU1, CYP9BU2 and CYP9R38). This *Drosophila* line showed an increase in resistance to thiacloprid compared to *Drosophila* lines containing individual genes, suggestive of an additive effect, although this result was not significant. There are a number of cases in which multiple P450s have been implemented in the insecticide resistance of insect pests. For example, Denecke et al. (2017) explored the resistance of a population of *D. melanogaster* from North Carolina to imidacloprid and found that both CYP6G1 and CYP6G2 played a role in imidacloprid resistance. Similarly, both CYP6BB2 and CYP6N12 have been implicated in the metabolism of imidacloprid by *Ae. Aegypti* (Riaz et al., 2013).

6.4.3 Summary

Taken together, these results seem to suggest that the tolerance of *O. bicornis* to a number of insecticides belonging to different insecticide classes is a result of a detoxification mechanism involving several P450 enzymes acting in concert. Of the tested P450s, CYP9BU1, which is a member of the most closely related subfamily to the eusocial thiacloprid-detoxifying CYP9Q subfamily, seems to be of particular importance as a generalist detoxification enzyme. These P450s exhibited broad substrate specificity as shown by their role in the detoxification of other insecticides and a number of P450 model substrates. Additionally, the contribution of CCEs and GSTs to xenobiotic detoxification by *O. bicornis* should be considered in future studies, although a substantially reduced number of GST members (12) was found in *Apis* compared to *Drosophila* (43) and *Anopheles* (37) genomes (Corona and Robinson, 2006).

Chapter seven: Patterns of P450 gene expression

Sample preparation for the differential expression of detoxification genes following neonicotinoid exposure was assisted by Emma Randall. RNAseq analysis detailed in this chapter was taught and assisted by Kumar Saurabh Singh.

7.1 Introduction

7.1.1 Relative expression of P450s in *O. bicornis* tissues associated with xenobiotic detoxification

Insect P450s have been found to be expressed in a variety of different tissues (Feyereisen, 2006), and their levels of expression at these sites can give an indication of their functional role (Seliskar and Rozman, 2007). P450s have been found to be highly expressed in components of the insect excretory system, further strengthening their role in insecticide detoxification (Chung et al., 2009). Characterising where these detoxifying P450s are expressed is an important aspect of understanding their capacity to protect *O. bicornis*. The excretory system of bees is predominantly made up of the hindgut and the Malpighian tubules, which are the insect equivalents of vertebrate kidneys (Ruiz-Sanchez and O'Donnell, 2015). Once ingested, xenobiotics can be temporarily stored in the crop before passing into the midgut, where they are taken up by midgut epithelial cells and metabolised. A small amount of the compound is transported back into the midgut lumen and across the membrane into the haemolymph. The majority of xenobiotics pass from midgut into the hindgut and Malpighian tubules where further metabolism takes place. These products then travel to the rectum where they are excreted (du Rand et al., 2017b).

P450s have also been found to be highly expressed in the brains of insects. For instance, the deltamethrin-metabolising CYP6BQ9 in *Tribolium castaneum* was found to be highly brain-specific (Zhu et al., 2010). Brain-specific expression may enable the rapid metabolism of insecticides that have passed through the blood-brain barrier, thus depleting the levels of insecticide able to bind to the target site. Neonicotinoids act on nAChRs in the bees central nervous system

(van der Sluijs et al., 2013) and as such the neonicotinoid-metabolising P450s identified in this study may be highly expressed in *O. bicornis* brains. During this study the expression levels of candidate P450s was explored in the brain, midgut and Malpighian tubules of *O. bicornis* females.

7.1.2 Differential expression of detoxification genes following neonicotinoid exposure

Exposure of insects to compromising conditions can lead to stress responses, often involving changes in gene expression levels (Moskalev et al., 2015). As mentioned previously, *O. bicornis* exhibits greater sensitivity to nitro- compared to cyano-substituted neonicotinoids and thus is likely to exhibit a greater stress response on exposure to nitro-substituted neonicotinoids. As detailed in chapter 6, this variation in sensitivity was found to be, at least in part, due to differences in the metabolic capabilities of P450 enzymes. Expression of insect P450s can be constitutive or induced upon exposure to insecticides (Li, 2007). For instance, Alptekin et al. (2016) carried out microarrays comparing the global gene expression levels of honey bees treated with thiacloprid and those treated with acetone. The expression of five P450s (*cyp6be1, cyp305d1, cyp6as5, cyp315a1*, and *cyp301a1*) and one CCE (CCE8) was found to be induced in bees treated with thiacloprid. It is not known whether the expression of *O. bicornis* P450s is constitutive or induced on exposure to neonicotinoids. Understanding whether the expression of metabolising P450s is constitutive or induced is important for the understanding of the metabolic defence of *O. bicornis*. The aim of this study was to explore whether detoxification enzymes can be induced on exposure to sublethal doses of cyano- (imidacloprid) and nitro- (thiacloprid) neonicotinoids, and whether this response varies between cyano- or nitro- substituted compounds. Additionally, the specific genes induced from exposure were identified.

7.2 Methods

7.2.1 Relative expression of P450s in *O. bicornis* tissues associated with xenobiotic detoxification

Adult female *O. bicornis* of a similar age were flash frozen in liquid nitrogen and

stored at -80°C prior to use. The head, thorax and abdomen of each bee were separated in a petri dish placed on dry ice using a scalpel. Tissues were isolated from bees as follows: prior to dissection the heads and abdomens were soaked in RNAlater®-ICE (Life technologies) (10 volumes of RNAlater®-ICE to 1 volume of tissue) at -20°C for at least 16 hours in order to prevent RNA degradation. Dissections were carried out using a stereomicroscope (Cole-Parmer) at 20x magnification. The head/abdomen was removed from the RNAlater®-ICE solution and secured with insect pins to a Black Sylgard Plate flooded with Ringer's solution (see appendix 1). The Ringer's solution was used to reduce the amount of osmosis taking place. Spring scissors with a 12 mm blade and forceps were used to remove the selected tissue (brain, midgut or Malpighian tubules) from the bee. The dissected tissues were placed back into the RNAlater®-ICE solution in individual 1.5 mL microcentrifuge tubes. All dissections were carried out in under 30 minutes to avoid degradation of RNA.

To extract RNA, each dissected tissue was ground up in 1.5 mL tubes placed on dry ice using a pre-cooled sterile polypropylene pellet pestle (Sigma-Aldrich). RNA was extracted using a Bioline Isolate II RNA Mini Kit (Bioline Reagents) following the manufacturer's recommended protocol. The quality and quantity of the RNA was assessed as described in section 2.2. First-strand cDNA was synthesised by reverse transcription as described in section 2.3.

Housekeeping genes were used as endogenous controls to enable the comparison of the relative expression of the target genes with the expression of reference genes. Preliminary qPCR tests were carried out as described in section 2.4.3 on a number of candidate housekeeping genes in order to identify genes that were stably expressed between tissues. Sequences of candidate genes were acquired from the annotated transcriptome described in chapter 5. The most consistently expressed housekeeping genes were found to be elongation factor α1 (EFα) and elongation factor γ1 (EFγ1) and thus these genes were selected. The expression of P450s and housekeeping genes in the different tissues were assessed by carrying out qPCR as detailed in section 2.4.3.

Data were analysed using Microsoft Excel, utilising the ΔΔCT method as

described by Pfaffl (2001). The geometric mean of the two housekeeping genes was used to normalise data. Data were further normalised to the midgut. Graphs were produced using GraphPad Prism 7.

7.2.2 Differential expression of detoxification genes following neonicotinoid exposure

Tested insecticides were dissolved in acetone to the highest concentration possible (to limit the amount of acetone consumed by the bees), before being diluted to the LD_{10} (based on data from chapter 3) of imidacloprid (0.0001 µg/bee) and thiacloprid (0.01 µg/bee) with 50% sucrose (w/v). For the duration of the bioassay females were housed individually in Nicot cages. Prior to commencing oral bioassays bees underwent a 24 hour 'training' period in the Nicot cages to allow them to learn to feed from the syringes. This was followed by a 16h starvation period to encourage feeding. 15 µL of the insecticide/sucrose solution was supplied orally to the bees in disposable plastic syringes. Control bees were fed 15 µL of a sucrose solution containing the same volume of acetone used to make up the insecticide/sucrose solutions. Once all of the solution had been consumed the bees were fed *ab libitum* with a 50% sucrose solution. For each condition four replicates were carried out comprising of five bees per replicate. After 24h the bees were snap frozen in liquid nitrogen and stored at -80ºC.

RNA was extracted from groups of five bees by grinding them to a fine homogenous powder in liquid nitrogen using a pestle and mortar and the Isolate RNA Mini Kit (Bioline) according to the product manual. The quantity and quality of RNA was checked as described in section 2.2.

RNA was used as a template for the generation of barcoded libraries (TrueSeq RNA library preparation, Illumina) which were sequenced across two lanes of an Illumina HiSeq2500 flowcell (100 bp paired end reads) to generate > 25M reads per replicate. Sequencing was carried out by Earlham Institute, Norwich, UK. The quality of the raw data was accessed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads were trimmed using Trim Galore

(https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The Tuxedo workflow was used to map the reads of each clone against the annotated reference genome using TopHat, to estimate gene expression with Cufflinks and test for differential expression between each treatment with Cuffdiff (Trapnell et al., 2012). All analyses were carried out using the cummeRbund package in R studio. The quality of the reads was assessed by looking at the distribution of reads (figure 41). The Gene Ontology (GO) terms, and KEGG terms associated with the significantly differentially expressed genes (DEGs) were explored using Blast2GO (https://www.blast2go.com/). Gene ontology comparison graphs were produced using GraphPad Prism 7. A venn diagram comparing different treatments was assembled using jvenn (http://genoweb.toulouse.inra.fr:8091/app/example.html).

7.3 Results

7.3.1 Relative expression of P450s in *O. bicornis* tissues associated with xenobiotic detoxification

The expression level of the majority of P450s tested were found to vary between tissues. The thiacloprid-metabolising *cyp9bu1* was found to be most significantly expressed in the Malpighian tubules relative to the brain and the midgut. Similarly, *cyp6as127*, *cyp6aq55, cyp6as151* and *cyp336a35* were found to be most highly expressed in the Malpighian tubules. On the other hand, *cyp9r39, cyp9dn1* and *cyp336a36*were comparatively more expressed in the brain. No significant differences were found in the expression of cyp9bu2, cyp9r1 and cyp9r38 amongst the brain, the Malpighian tubules and the midgut (figure 40).

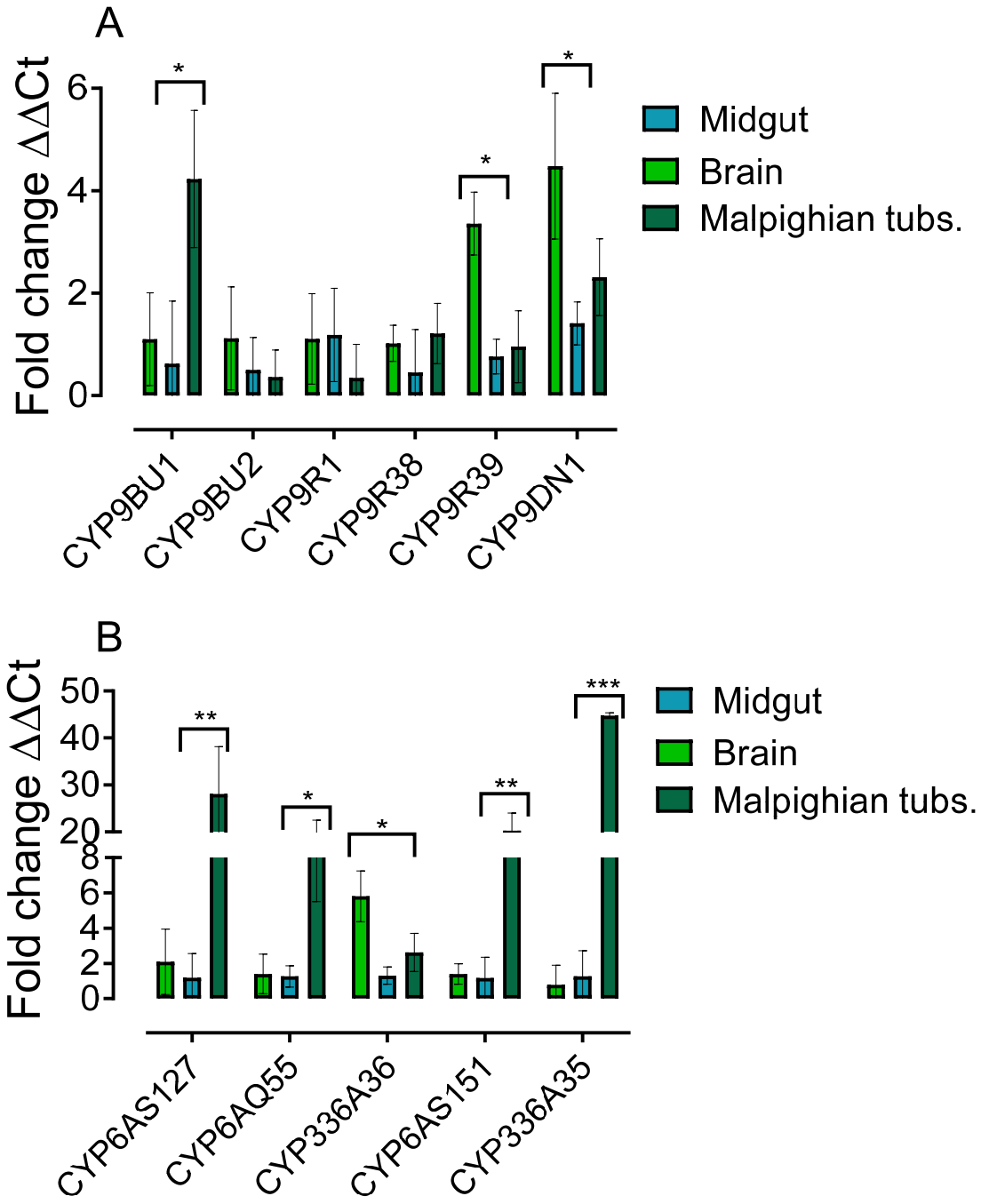


Figure 40. Relative expression of P450s belonging to the CYP9 (A) and CYP6 (B) families in the midgut, brain and Malpighian tubules. Data are normalised to the housekeeping genes elongation factor α and elongation factor γ1 and additionally to the midgut. Data points are mean values (n=3, ±95% CI) Significant differences of expression levels in tissues compared to the expression level in the midgut are denoted by * $p<0.05$, ** $p<0.005$, *** $p<0.0005$; paired t-test).

7.3.2 Differential expression of detoxification genes following neonicotinoid exposure

The global gene expression response was found to be low for both treatments. In total, 35,188 genes were identified as expressed. A greater response in terms of gene expression changes was detected in bees exposed to a sublethal dose of imidacloprid compared to thiacloprid. 79 significantly DEGs were identified in imidacloprid treatments, of which, 27 were upregulated and 52 were downregulated (table 12). In comparison, 34 significantly DEGs were detected in thiacloprid treatments, of which 16 were upregulated and 18 were downregulated (table 13). 20 genes were found to be in common between the two treatments. 67% of overexpressed genes were enriched for GO terms in imidacloprid treatments, and 74% in thiacloprid treatments. Based on the GO terms for biological processes, 22% of these genes were enriched for metabolic processes in imidacloprid treatments and 39% in thiacloprid treatments. Based on the GO terms for molecular function, 41% of DEGs were enriched for catalytic activity in imidacloprid treatments and 53% for thiacloprid treatments, including oxidoreductase, transferase and hydrolase activity (figure 44). The majority of DEGs in both imidacloprid and thiacloprid treatments were found to be associated with the membrane in terms of cellular components (24% and 35% respectively). KEGG pathways were used to assign functional annotations to the DEGs. A small number of DEGs were enriched for KEGG pathways. Of interest was an alcohol dehydrogenase gene that was enriched for 'detoxification of xenobiotics by P450s' (g5989.t1). This gene was significantly upregulated in imidacloprid treatments. However, no P450s were found to be significantly upregulated in either treatment.

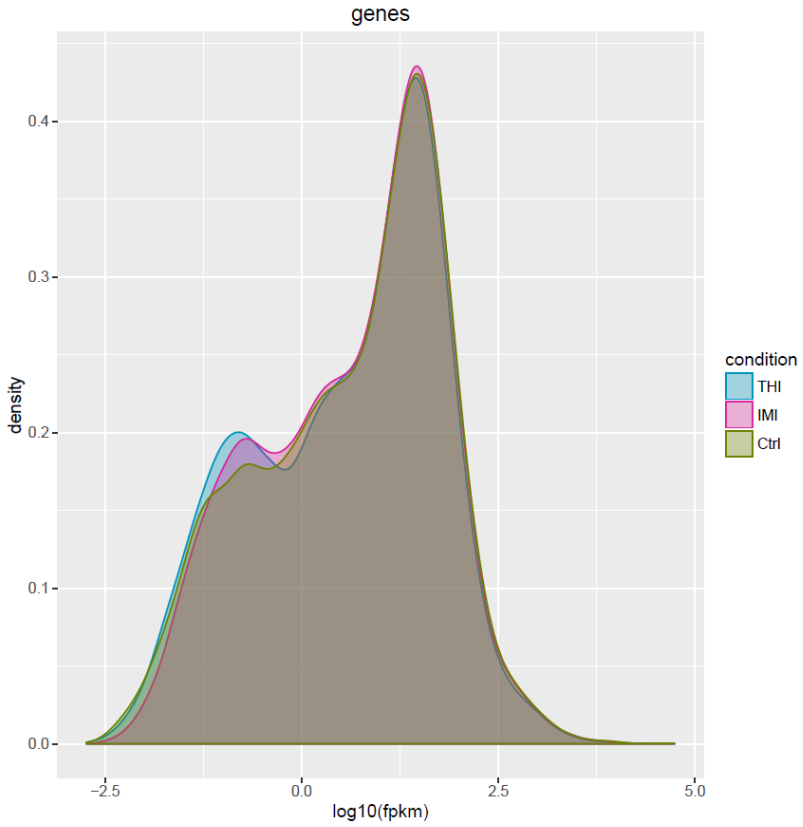


Figure 41. Quality control check: The distribution of *O. bicornis* RNAseq reads from different treatments.

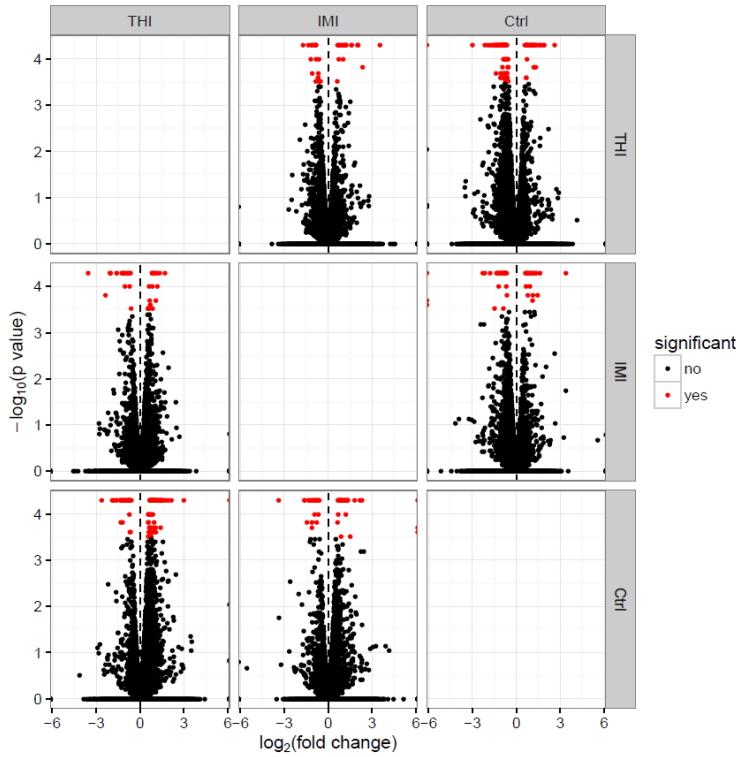


Figure 42. A volcano plot showing log10 fragments per kilobase of transcript per million mapped reads (fpkm) of differentially expressed genes (red) in different treatment combinations.

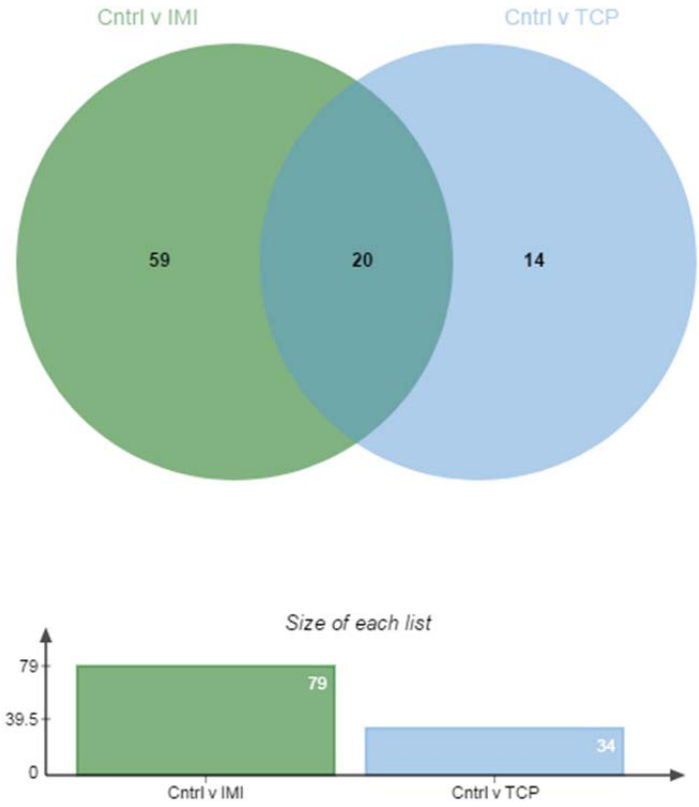


Figure 43. Venn diagram showing the number of differentially expressed genes in each treatment comparison.

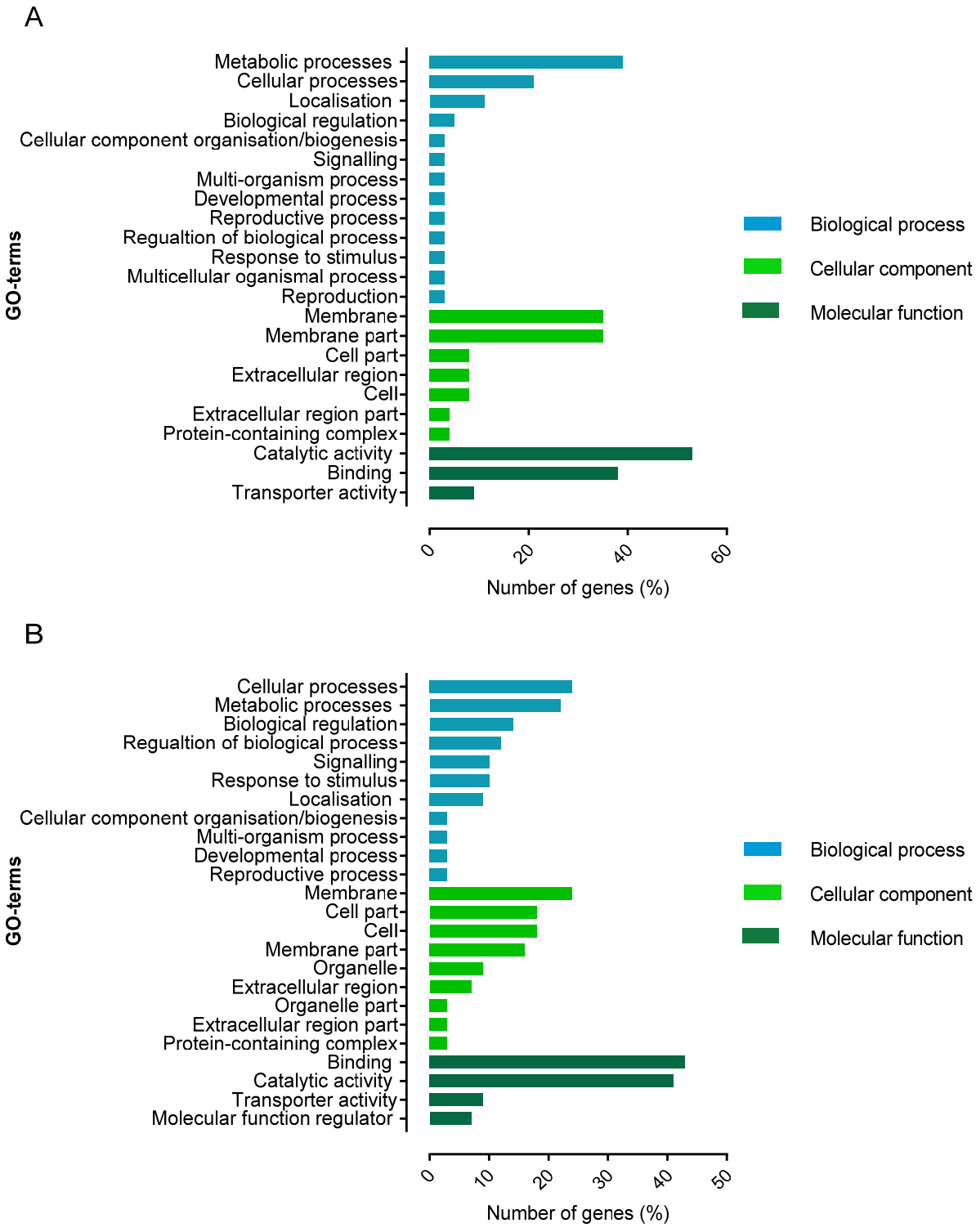


Figure 44. GO-enrichment analysis of differentially expressed genes (DEGs) in *O. bicornis* treated with thiacloprid (A) and imidacloprid (B) after 24 hours. Blue bars represent DEGs enriched for biological process, green bars represent DEGs enriched for cellular component, and teal bars represent DEGs enriched for molecular function.

Gene ID	Fold-change	Log2 (foldchange)	Q value	Blast annotations	Description
g2479	11.66	3.51	0.01	uncharacterized protein LOC100876166 [*Megachile rotundata*]	Esterase
g2478	4.17	2.05	0.01	uncharacterized protein LOC100876166 [*Megachile rotundata*]	Esterase
g29168	4.03	2.01	0.01	predicted protein, partial [*Nematostella vectensis*]	Apidaecins- inducible peptide antibiotics
g32438	4.03	2.01	0.01	hypothetical protein WH47_07919 [*Habropoda laboriosa*]	Involved in immune response
g4392	3.04	1.60	0.01	cytochrome b5-related protein-like [*Megachile rotundata*]	Cytochrome b5-related
g9698	3.01	1.58	0.01	transferrin [*Megachile rotundata*]	Transferrin
g30471	2.35	1.23	0.01	protein lethal(2)essential for life-like [Megachile	Involved in stress response

				rotundata]	
g31064	2.18	1.12	0.01	carboxypeptidase Q-like isoform X1 [*Megachile rotundata*]	Enzyme that hydrolyses peptide bonds
g29351	2.14	1.09	0.01	L-lactate dehydrogenase-like [*Megachile rotundata*]	Coverts NAD+ to NADH and back
g3206	1.99	0.99	0.01	cytochrome b561 domain-containing protein 1-like [*Megachile rotundata*]	Cytochrome b5-related
g33652	1.83	0.87	0.01	uncharacterized protein LOC100881220 [Megachile rotundata]	Uncharacterised
g4165	1.68	0.74	0.01	venom allergen 3-like [*Megachile rotundata*]	Cysteine-rich secretory proteins
g6850	1.66	0.73	0.01	heat shock 70 kDa protein cognate 4 isoform X1 [*Megachile rotundata*]	Involved in stress response
g914	1.65	0.71	0.02	uncharacterized protein LOC100864565 [*Apis florea*]	Uncharacterised

g2055	1.56	0.63	0.01	Terminal uridylyltransferase 7 [*Dufourea novaeangliae*]	Involved in stress response
g33840	0.31	-1.71	0.01	PREDICTED: ornithine decarboxylase 2-like isoform X1 [*Megachile rotundata*]	Decarboxylation of ornithine
g5563	0.39	-1.34	0.01	PREDICTED: serine protease nudel isoform X2 [*Megachile rotundata*]	Involved in the activation of a protease cascade
g33322	0.44	-1.18	0.02	PREDICTED: facilitated trehalose transporter Tret1-like isoform X2 [*Megachile rotundata*]	Trehalose transporters
g15329	0.45	-1.14	0.01	PREDICTED: elongation of very long chain fatty acids protein 1-like [*Dufourea novaeangliae*]	Elongation of fatty acids
g8515	0.46	-1.10	0.01	PREDICTED: nose resistant to fluoxetine protein 6-like isoform X1 [*Megachile rotundata*]	Uptake of xenobiotic compounds
g27717	0.47	-1.08	0.04	PREDICTED: piggyBac transposable element-	Mobile genetic element that can transpose

				derived protein 4-like [*Polistes dominula*]	between vectors and chromosomes
g23031	0.52	-0.94	0.01	PREDICTED: nose resistant to fluoxetine protein 6-like isoform X1 [*Megachile rotundata*]	Uptake of xenobiotic compounds
g6999	0.54	-0.88	0.01	PREDICTED: uncharacterized protein LOC100876107 [*Megachile rotundata*]	Uncharacterised
g5009	0.55	-0.86	0.01	PREDICTED: uncharacterized protein LOC100880123 [*Megachile rotundata*]	Uncharacterised
g2078	0.55	-0.85	0.05	Protein kinase DC2 [*Habropoda laboriosa*]	Kinase enzyme involved in phosphorylation
g34065	0.55	-0.84	0.01	PREDICTED: sodium-dependent nutrient amino acid transporter 1-like [*Megachile rotundata*]	Uncharacterised
g5456	0.56	-0.83	0.02	PREDICTED: farnesyl pyrophosphate synthase isoform X3 [*Megachile rotundata*]	Farnesyl pyrophosphate synthase
g31841	0.56	-0.82	0.01	PREDICTED:	Uncharacterised

				uncharacterized protein LOC100874903 [*Megachile rotundata*]	
g32954	0.62	-0.69	0.04	PREDICTED: fibrillin-2-like isoform X1 [*Megachile rotundata*]	Glycoprotein
g1430	0.62	-0.69	0.04	PREDICTED: phenoloxidase 2 [*Megachile rotundata*]	Involved in immune response

Table 12. Top 15 upregulated and downregulated gene in *O. bicornis* treated with thiacloprid including fold change, log fold change, BLAST annotation and suggested description based on a literature search.

Gene ID	Fold-change	Log2(fold change)	Q value	Blast annotation	Description
g29168	6.12	2.61	0.01	predicted protein, partial [*Nematostella vectensis*]	Apidaecins- inducible peptide antibiotics
g32741	3.74	1.90	0.01	uncharacterized protein LOC100880045 isoform X1 [*Megachile rotundata*]	Uncharacterised protein
g31521	3.51	1.81	0.01	uncharacterized protein LOC105664198 [*Megachile rotundata*]	Uncharacterised protein
g30471	3.11	1.64	0.01	protein lethal(2)essential for life-like [*Megachile rotundata*]	Essential for life-like
g4165	3.00	1.59	0.01	venom allergen 3-like [*Megachile rotundata*]	Stress response

g914	2.53	1.34	0.01	uncharacterized protein LOC100864565 [*Apis florea*]	Uncharacterised protein
g32438	2.51	1.33	0.01	hypothetical protein WH47_07919 [*Habropoda laboriosa*]	Involved in immune response
g2479	2.50	1.32	0.02	uncharacterized protein LOC100876166 [*Megachile rotundata*]	Esterase
g29351	2.49	1.32	0.01	L-lactate dehydrogenase-like [*Megachile rotundata*]	Lactate dehydrogenase
g6029	2.31	1.21	0.01	chymotrypsin inhibitor-like [*Megachile rotundata*]	Digestive enzyme inhibitor
g2245	2.28	1.19	0.02	uncharacterized protein LOC100877442 [*Megachile rotundata*]	Uncharacterised protein
g30763	2.24	1.17	0.01	pancreatic triacylglycerol lipase-like [*Megachile rotundata*]	Hydrolyses dietary fat
g29252	2.19	1.13	0.01	probable G-protein coupled receptor No9 [*Megachile rotundata*]	G-protein coupled receptor
g6850	2.07	1.05	0.01	heat shock 70 kDa protein cognate 4 isoform X1 [*Megachile rotundata*]	Stress response
g1945	2.02	1.01	0.01	putative helicase mov-10-B.1 [*Megachile rotundata*]	Helicase
g5701	0.12	-2.99	0.01	PREDICTED: 4-coumarate--CoA ligase 1-like [*Megachile rotundata*]	Ligase
g27717	0.22	-2.14	0.01	PREDICTED: piggyBac transposable element-derived protein 4-like [*Polistes dominula*]	Mobile genetic element that can transpose between vectors and chromosomes
g19074	0.26	-1.92	0.01	PREDICTED: elongation of very long chain fatty acids protein 1-like	Elongation of fatty acids

				[*Dufourea novaeangliae*]	
g8515	0.29	-1.76	0.01	PREDICTED: nose resistant to fluoxetine protein 6-like isoform X1 [*Megachile rotundata*]	Uptake of xenobiotic compounds
g23893	0.33	-1.59	0.01	PREDICTED: LOW QUALITY PROTEIN: uncharacterized protein LOC105663869 [*Megachile rotundata*]	Uncharacterised
g5456	0.34	-1.53	0.01	PREDICTED: farnesyl pyrophosphate synthase isoform X3 [*Megachile rotundata*]	farnesyl pyrophosphate synthase
g12942	0.37	-1.41	0.01	PREDICTED: cytochrome P450 6B1-like [*Megachile rotundata*]	Cytochrome P450
g34176	0.37	-1.40	0.01	PREDICTED: purine nucleoside phosphorylase-like isoform X1 [*Megachile rotundata*]	catalyze the phosphorolytic breakdown of the *N*-glycosidic bond in the beta-(deoxy)ribonucleoside molecules
g1386	0.38	-1.39	0.04	PREDICTED: 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1 [*Apis mellifera*]	Mediates production of messenger molecules
g15329	0.39	-1.34	0.01	PREDICTED: elongation of very long chain fatty acids protein 1-like [*Dufourea novaeangliae*]	Elongation of fatty acids
g34176	0.39	-1.34	0.01	PREDICTED: purine nucleoside phosphorylase-like isoform X1 [*Megachile rotundata*]	glycosyltransferase
g23031	0.42	-1.24	0.01	PREDICTED: nose resistant to fluoxetine protein 6-like isoform X1	Uptake of xenobiotic

				[*Megachile rotundata*] >gi|805757567|ref|XP_012146697.1| PREDICTED: nose resistant to fluoxetine protein 6-like isoform X1 [*Megachile rotundata*]	compounds
g29296	0.43	-1.21	0.01	PREDICTED: potassium channel subfamily T member 2 [*Megachile rotundata*]	Involved in potassium channel
g8960	0.47	-1.08	0.04	PREDICTED: GTPase-activating Rap/Ran-GAP domain-like protein 3 isoform X3 [*Bombus impatiens*]	Hydrolase enzyme
g9923	0.4	-1.08	0.01	PREDICTED: high-affinity choline transporter 1 [*Megachile rotundata*]	Choline transporter

Table 13. Top 15 upregulated and downregulated gene in *O. bicornis* treated with imidacloprid including fold change, log fold change, BLAST annotation and suggested description based on a literature search.

7.4 Discussion

7.4.2 Relative expression of P450s in *O. bicornis* tissues associated with xenobiotic detoxification

The thiacloprid-metabolising P450 *cyp9bu1* was found to be most highly expressed in the Malpighian tubules, further supporting its prominent role in insecticide detoxification. The comparably greater expression of this enzyme in the Malpighian tubules may greatly enhance the ability of *O. bicornis* to detoxify insecticides. Similarly, the thiacloprid-metabolising P450 *cyp9q3* in *A. mellifera* was found to be most highly expressed in the Malpighian tubules (Manjon et al., 2018).

cyp6aq55, a prominent metaboliser of nicotine, was also found to be significantly expressed in the Malpighian tubules. As a caterpillar, the tobacco hornworm, *Manduca sexta*, can live entirely on tobacco. The Malpighian tubules of this species are able to remove nicotine from the haemolymph at high rates (Gaertner et al., 1998), highlighting the importance of the Malpighian tubules in nicotine metabolism.

cyp9dn1 and *cyp336a36* were comparatively more highly expressed in the brain, suggesting that they may play a role in the defence of nAChRs in the brain against insecticides that have passed through the blood-brain barrier.

Overall, the expression of these detoxifying P450s in the brain, midgut and Malpighian tubules was found to vary, with the majority found to be most highly expressed in excretory tissues, further strengthening the role of these P450s in the detoxification of xenobiotics by *O. bicornis*.

7.4.2 Differential expression of detoxification genes following neonicotinoid exposure

Exposure of *O. bicornis* to sublethal doses of imidacloprid and thiacloprid revealed a greater response in terms of changes in gene expression on imidacloprid exposure, further supporting the greater toxicity of imidacloprid

compared to thiacloprid. GO analysis of DEGs revealed terms mainly associated with a stress response but DEGs were also enriched for metabolic processes including oxidoreductase, transferase and hydrolase activity. No significant overexpression of P450s was observed in either treatment, suggesting that expression of P450s in *O. bicornis* is constitutive rather than induced. Although cytochrome b5, which can act as an electron carrier for P450s, was found to be upregulated in both comparisons. This is consistent with the differential tissue expression of *O. bicornis* P450s, as differentially expressed genes in tissues are not normally inducible (Feyereisen, 2006).

In contrast to this study, previous studies on *A. mellifera* have found that P450 pathways can be induced in response to xenobiotics. For example, similar to Alptekin et al. (2016), a study by Shi et al. (2017) exposed *A. mellifera* workers to thiamethoxam and found that *cyp9as5* was overexpressed, suggesting that this P450 is involved in xenobiotic detoxification. During this study *A. mellifera* was exposed to thiamethoxam over a 10-day period. As *O. bicornis* were only exposed to the neonicotinoids for 24 hours it may be that this period of exposure was not long enough or the sublethal dose of insecticide applied was not high enough to induce overexpression of P450s. Additionally, the diet fed to bees has been found to influence the upregulation of P450s in bees (Mao et al., 2011).

Similar to this study, Johnson et al. (2012), found that phenobarbital, a well-known inducer of P450s, failed to induce the expression of any P450 genes in a microarray analysis of *A. mellifera* and concluded that bees do not seem to be capable of inducing P450s in response to xenobiotics. Further studies are needed, particularly on other solitary bee species, in order to fully assess whether P450 expression is constitutive or induced on exposure to insecticides in different bee species.

In summary, exposure of *O. bicornis* to SPMs has resulted in the evolution of a natural detoxification mechanism that allows tolerance of low levels of SPMs in nectar and pollen. Furthermore, *O. bicornis* can utilise this mechanism to detoxify similarly structured synthetic neonicotinoids. This detoxification mechanism involves a number of generalist P450s which were predominantly

found to be highly expressed in the Malpighian tubules, highlighting their importance in xenobiotic detoxification. Exposure of *O. bicornis* to sublethal doses of neonicotinoids resulted in a greater response in terms of DEGs to the nitro-substituted neonicotinoid imidacloprid compared to the cyano-substituted neonicotinoid thiacloprid. However, no P450s were found to be overexpressed on exposure to either compound, suggesting that the expression of P450s in *O. bicornis* is constitutive rather than induced.

Chapter 8: General discussion

This chapter aims to summarise the key findings of this PhD thesis, placing them in the context of the wider literature. The applications of this research and suggestions for future work are also discussed.

8.1 Key findings

O. bicornis exhibits significant differences in sensitivity to cyano- and nitro-substituted neonicotinoids (chapter 3)

Acute toxicity bioassays revealed that like honey bees and bumblebees, *O. bicornis* exhibits significant differences in sensitivity to nitro- (imidacloprid) and cyano- (thiacloprid) substituted neonicotinoids, with thiacloprid exhibiting >2,000-fold less toxicity than imidacloprid. Furthermore, thiacloprid sensitivity, but not imidacloprid sensitivity, was greatly increased upon pre-treatment of the P450 inhibitor PBO, suggesting that P450s play a role in determining the sensitivity of *O. bicornis* to neonicotinoid insecticides.

With the exception of bee species, there are very few examples of intrinsic differences in the sensitivity of insects to insecticides. However, differences in neonicotinoid sensitivity have been observed in resistant insect pest species, with some species exhibiting a greater resistance to nitro- compared to cyano-substituted neonicotinoids. For example, the Kushima clone of the cotton aphid, *Aphis gossypii* Glover, was found to show a greater resistance to imidacloprid compared to acetamiprid. Hirata et al. (2017) explored the role of the R8IT mutation (a nAChR mutation that has previously been attributed to neonicotinoid resistance) in neonicotinoid sensitivity. It was found that imidacloprid exhibited a reduced affinity for the nAChR compared to acetamiprid, providing a potential explanation for this sensitivity difference.

In comparison, a number of insect pest species do not exhibit large differences in sensitivity to insecticides within the same class. For example, the brown plant hopper, *Nilaparvata lugens*, displays similar levels of sensitivity to imidacloprid

and thiacloprid with LD_{50} values of 0.32 mg/hopper and 1.6 mg/hopper respectively (Jeschke and Nauen, 2008).

Differential sensitivity of *O. bicornis* to cyano- and nitro- substituted neonicotinoids is not due to differences in binding affinity for the nAChR or rate of cuticular penetration (chapter 4)

No significant differences were found in the binding affinity of imidacloprid and thiacloprid to *O. bicornis* nAChRs, demonstrating that differences in the binding affinity of imidacloprid and thiacloprid for nAChRs does not explain the marked variation in bee sensitivity to these compounds.

These results are consistent with a previous study by Manjon et al. (2018) who found no significant difference in the binding affinity of these neonicotinoids to honey bee and bumblebee nAChRs (Manjon et al., 2018). However, a previous study by Wu et al. (2017) explored the molecular basis of the reduced sensitivity of the common eastern bumblebee *B. impatiens* to tau-fluvalinate, a pyrethroid that acts on the sodium channel. It was found that the sodium channel $BiNa_v$ 1-1 is highly sensitive to six commonly used pyrethroids (e.g. deltamethrin) but is selectively resistant to tau-fluvalinate. Specifically, it was found that three residues (T841, V926, and F1525) that are conserved across a number of bee species are of particular importance to determining the sensitivity to pyrethroids. Due to this, differential binding affinity as a contributing factor to differences in insecticide sensitivity observed in other insecticide classes should not be ruled out in future studies.

This phenomenon has also been observed between different species. For example, the green peach potato aphid *M. persicae* has been found to be selectively sensitive to imidacloprid compared to the pond wolf spider, *Pardosa pseudoannulata*, which is an important predatory enemy of this species. The Ppβ1 subunit of the wolf spider has a high identity to β1 subunits in insect species, however amino acid differences are found within loops D, E and F of the Ppβ1 subunit. Introduction of these loops into the corresponding regions of rat β2 subunits and expression of these hybrid nAChRs in *Xenopus* oocytes resulted in a decrease in imidacloprid sensitivity (as denoted by a right-ward shift in dose-response curves), demonstrating that these amino acid differences are key to the neonicotinoid selectivity conferred between these two species

(Song et al., 2009). These data provide important information for the selective targeting of insect pests whilst avoiding detrimental effects to their natural predators.

Similar rates of penetration of imidacloprid and thiacloprid were observed through the cuticle of *O. bicornis*, suggesting that the rate of insecticide penetration does not play a role in neonicotinoid sensitivity. However, acetamiprid was found to have a greater rate of penetration compared to imidacloprid and thiacloprid. This superior ability may have been due to its acyclic, open-chained structure compared to imidacloprid and thiacloprid which possess a heterocyclic ring. This structural knowledge may be useful in assisting the design of bee-safe insecticides.

O. bicornis and most other solitary bee species lack the CYP9Q subfamily found to break down neonicotinoids in eusocial bee species (chapter 5). Instead, detoxification of neonicotinoids is carried out by members of the CYP9BU subfamily (chapter 6)

As discussed in chapter 5, the *O. bicornis* CYPome, along with the CYPomes of most other solitary bee species, were found to lack the CYP9Q subfamily, which has previously been implicated in the detoxification of thiacloprid in honey bees and bumblebees. Members of the most closely related subfamily to CYP9Q, CYP9BU, was found to be predominant metabolisers of a number of insecticides, suggestive of an important role as generalist detoxification enzymes. These enzymes displayed a greater binding affinity to thiacloprid compared to imidacloprid. Furthermore, transgenic *Drosophila* expressing CYP9BU1 displayed significant resistance to thiacloprid but not imidacloprid compared to the control line. Taken together, these results could explain, at least in part, the reduced sensitivity of *O. bicornis* to thiacloprid.

It can be speculated that bee species that do not employ either the CYP9Q or CYP9BU subfamilies may be more sensitive to certain insecticides. Indeed, recent bioassays on *M. rotundata* have shown a substantially greater sensitivity to thiacloprid compared to *O. bicornis* and other species (LD_{50} of 0.014 µg/bee compared to >100 µg/bee respectively, Angie Hayward, personal

communication). These genetic differences could have implications on the use of *O. bicornis* as a model species during toxicity testing for the registration of new insecticides (European Food Safety, 2013).

O. bicornis is equipped with a natural detoxification mechanism that can be utilised to detoxify synthetic compounds (chapter 6)

O. bicornis microsomes displayed a substantial capability to metabolise the secondary plant metabolite nicotine and incubation of recombinant P450s with nicotine identified CYP6AQ55 as a major nicotine metabolising enzyme. Furthermore, as detailed in chapter 6 CYP6AQ55 was also able to metabolise the neonicotinoids imidacloprid, thiacloprid and acetamiprid. These results provide preliminary evidence to suggest that *O. bicornis* may be equipped with a pre-existing detoxification mechanism, possibly due to exposure to secondary plant metabolites in their diet, that can be utilised to detoxify similarly structured synthetic insecticides. Further research is required to confirm this suggestion. For example, similar to Mao et al. (2009), it would be interesting to look at the metabolism of quercetin by both microsomes and recombinant P450s. This experiment was attempted during this PhD but unfortunately the metabolic standard needed for LC-MS analysis was not available.

Expression of P450s in *O. bicornis* are constitutive and tissue-specific (chapter 7)

Exposure of *O. bicornis* to sublethal doses of imidacloprid and thiacloprid did not induce any P450 expression, suggesting that the expression of insecticide-metabolising P450s is constitutive. Furthermore, selected P450s were found to be differentially expressed between tissues, with some P450s highly expressed in the Malpighian tubules, a primary site of xenobiotic detoxification, or in the brain of *O. bicornis*, which contains high concentrations of nAChRs.

The tissue-specific expression of genes is commonly regulated by transcription factors and is important in determining the toxicodynamics of insecticides (Giraudo et al., 2010). Giraudo et al. (2010) carried out a similar study utilising

both of these approaches. Firstly, the tissue-specific expression of the insecticide-detoxifying CYP6A2 in *D. melanogaster* was explored, and secondly, the induction of other P450s was determined, placing CYP6A2 in the wider context of the whole P450 family. Similar to the prominent thiacloprid-metaboliser CYP9BU1 identified in *O. bicornis*, this analysis revealed that *cyp6a2* is highly expressed in the Malpighian tubules and mid gut of *D. melanogaster*. Furthermore, it was found that only a third of *D. melanogaster* P450s could be induced by xenobiotics, and the majority are not inducible.

8.2 Practical applications of research

8.2.1 Knowledge contributions

8.2.1.1 The rational design of bee-safe insecticides

During this study it was found that *O. bicornis* was more sensitive to some neonicotinoids than others, with the conclusion that their sensitivity to these compounds seems to depend on the presence of the nitroguanidine pharmacophore and its retention during biotransformation (i.e. OH-imidacloprid is more toxic than OH-thiacloprid on topical application). These data provide a more detailed understanding of the chemical structures that bees are able to break down effectively. This information could be used to help design insecticides with a more targeted approach, that are of greater specificity to insect pests.

8.2.2 Development of *in vitro* and *in vivo* tools

8.2.2.1 Bee sensitivity screening tool of new insecticides

During this study two different approaches were utilised to functionally characterise candidate P450 enzymes. Namely, by expressing P450s in an insect cell line followed by incubation with thiacloprid and imidacloprid, and by inserting P450s into *Drosophila* and assessing their sensitivity to these insecticides. In addition to functionally characterising genes of interest, a further aim of this approach was to compare the feasibility of these two methods in

generating a library of detoxifying P450s enzymes, which could be developed into a screening tool to quickly assess the capabilities of bees to detoxify new insecticides.

The development of a novel insecticide, from initial activity testing to registration approval can take up to ten years, and cost around $200,000,000 (Sparks and Nauen, 2015). This tool could be utilised early on in the development of insecticides to prevent the development of insecticides deemed unsafe to bees further down the line. Indeed, during the development of new pharmaceutical drugs key P450 enzymes in humans such as CYP3A4 are actively screened to prevent adverse reactions (Feyereisen, 2018). Furthermore, the active period of *O. bicornis* tends to be between 2 and 3 months (Fliskiewicz et al., 2015) and studies have found that this period cannot be extended without incurring detrimental effects on the developing bees (Dmochowska et al., 2013). This makes the testing of *O. bicornis* and other solitary bee species difficult. In contrast, *D. melanogaster*/ insect cell lines can be used all year round and so this tool could be used as a proxy to initially test new compounds.

Strengths and weaknesses were found for both methods. Using an insect cell line to express recombinant P450s was found to be more time consuming and potentially more costly but also required less maintenance and generated a more accurate output compared to transgenic *Drosophila* lines. Due to this, the insect cell line approach may be more viable.

8.2.2.2 Using detoxifying P450s as genetic markers for insecticide sensitivity in closely related species

For an insecticide to be successfully registered for use it must show a high efficacy to insect pests whilst ensuring that there are no inadvertent effects on the environment or beneficial insects such as bee pollinators. As such, all insecticides must undergo rigorous testing, including toxicity testing on a number of model bee species such as *A. mellifera* and *B. terrestris*. It has previously been found that extrapolating toxicity data to other bee species of varying ecology (e.g. different behaviours and/or body mass) (Cresswell et al., 2012) is not viable. However, there are currently over 20,000 described species

of bee (Linsley, 1958, Michener, 1974), making the testing of insecticides on every bee species unrealistic. The insecticide-detoxifying P450s identified during this project could be used as genetic markers to try and predict and prevent the inadvertent harmful effects an insecticide may have on a closely-related species. For example, phylogenetic analysis of the sequenced CYPomes of *A. dorsata*, *A. florea*, and *A. cerana* revealed that they all contain P450s belonging to the CYP9Q subfamily, which was found to be involved in thiacloprid metabolism in *A. mellifera*. These P450s share a high percentage sequence identity (~85%), indicating that this subfamily is well conserved. Thus, species in which this subfamily is present may have a similar ability to metabolise thiacloprid. In order to confirm this, insecticide toxicity bioassays would need to be carried out and compared on these closely-related species.

8.2.2.3 Using detoxifying P450s as genetic markers for selecting insecticide-tolerant bees

Biomarkers are commonly used to selectively breed bee colonies that are more resistant to the parasitic mite *Varroa destructor* (Guarna et al., 2016). These biomarkers have been found to be differentially expressed between susceptible and tolerant bee colonies (Wang, 2016). In the same way, the detoxification genes identified in this study could be used as biomarkers for the selection of bees that are more tolerant to insecticides, although this would be restricted to bee pollination in green houses due to the effects of outbreeding.

8.3 Future work

- **Functional characterisation of other P450s, GSTs and CCEs**
  During this study 11 P450 genes belonging to six subfamilies were functionally characterised. However, members of other subfamilies such as the CYP9P subfamily could be involved in the detoxification of xenobiotics that thus should be characterised. Additionally, GSTs and CCEs which have previously been found to be involved in xenobiotic detoxification could be characterised.

- **Inclusion of cytochrome b5 in the expression of recombinant P450s**
  The expression of recombinant P450s in insect cell lines was carried out by co-infecting High Five™ cells with baculovirus containing the P450 of interest and baculovirus containing CPR. However as mentioned previously, cytochrome b_5 has also been shown to play a role in P450 catalysis (Feyereisen, 2012) and thus it would be interesting to see if the inclusion of b_5 has an effect on P450 activity.

- **Knock-down of detoxifying P450s using RNA interference**
  RNA interference (RNAi) can be used to suppress the expression of a gene of interest, allowing the linking of phenotype and gene function (Scott et al., 2013). RNAi could be used to suppress the expression of a key detoxifying P450 identified in this study such as *cyp9bu1*, to assess whether the suppression of these genes results in an increased sensitivity of *O. bicornis* to thiacloprid.

- **Expression of detoxifying P450s in different life-stages of *O. bicornis***
  Studies have found that P450s can be differentially expressed during the life stages of an insect (Yu et al., 2015). Thus, *O. bicornis,* may be more/less susceptible to insecticides during different life stages depending on the expression levels of detoxifying P450s. Thus, the expression of P450s in bees during the larval, diapause, and postdiapause quiescence stages as well as in recently emerged bees could be tested. This would provide important information for the assessment of the sensitivity of *O. bicornis* to insecticides.

- **Computational modelling of detoxifying P450s**
  Computational modelling of the detoxifying P450s identified during this study could be used to confirm P450-substrate interactions and resultant metabolites. It could also be used to explore the inhibitory interactions of select insecticides with azole fungicides (see chapter 3). Molecular docking could be utilised as a prediction tool to assess the possible interactions (i.e. metabolism and inhibition) of detoxifying P450s and new insecticide compounds (Arimoto, 2006). Additionally, this research

provides the opportunity to identify the essential amino acid residues located in the active site of these important P450s in both *O. bicornis* and other bee species. This would help identify the structural moieties that are important for binding of different compounds. These results could then be confirmed by site-directed mutagenesis studies (Song et al., 2009).

8.4 Concluding remarks

Whilst there is no doubt that pollinators are increasingly threatened, extensive media coverage of the decline of pollinators has coined the term ‘global pollination crisis’, leading to some additional debate over how extensive this ‘crisis’ is (Ghazoul, 2005). The documented cases of decline seem to be limited to commercialised honey and bumble bees and thus additional data of the population levels of wild bee species are needed (Garibaldi et al., 2009, Ghazoul, 2005).

The reason(s) for this decline are still debated but is likely a result of a number of factors. The effect of insecticides on bee health has received considerable attention comparative to other factors, and concern about the inadvertent harmful effects of neonicotinoids has led to a 2-year moratorium on the use of seed treatments containing clothianidin, imidacloprid and thiamethoxam on certain crops within the EU (European Commission, 2013). Recently this ban has been extended to include a total ban on the outdoor use of these insecticides (European Commission, 2018). Whilst ensuring the safety of pollinators is of utmost importance, all possible implications of this ban must be considered. The implications of this ban will party depend on the response of farmers in their pest management practices (Kathage et al., 2018). Chemical insecticides are a vital tool for many farmers. With the number of cases of insecticide resistance continuing to rise, these tools are becoming more limited. Banning such insecticides without providing alternatives could lead to the use of older, more harmful insecticides. It could also cause farmers to stop growing certain crops such as oil seed rape that are a vital food source to bees. These responses may be more detrimental to the pollinators for which the ban was brought in to protect.

Although chemical insecticides have been tarred with a bad name this research and others shows that through the understanding of selectivity mechanisms, the development of insecticides that exhibit a high efficacy to insect pests whilst still ensuring the safety of pollinators and natural predators may still be possible.

Appendices

Appendix 1: Media, buffer and fly food recipes

1.1 LB (Luria-Bertani) medium and agar, pH adjusted to 7.0 with NaOH

Component	Amount (g)
Tryptone	10
Yeast Extract	5
NaCl	0.5
Bacto-agar (for LB agar)	15

Distilled H_2O to 1 litre

1.2 Potassium-Phosphate Buffer [0.1 M], pH adjusted to 7.6 with H_3PO_4

Component	Amount (g)
K_2HPO_4 [100mM]	87
Saccharose **[200 mM]**	34.2
EDTA (Ethylenediaminetetraacetic acid) **[1 mM**]	0.186

Distilled H_2O to 5 litres

1.3 Homogenisation buffer

Component	Amount (g)
DTT; 1,4-Dithio-DL-threitol **[1 mM]**	0.308
Saccharose **[200 mM]**	34.2
EDTA (Ethylenediaminetetraacetic acid) **[1 mM**]	0.186

Potassium-Phosphate Buffer [0.1 M] to 500 mL

1.4 Buffer R

Component	Amount (g)
DTT; 1,4-Dithio-DL-threitol **[1 mM]**	0.0154
Glycerol [5%]	5
EDTA(Ethylenediaminetetraacetic acid)**[0.1 mM**]	0.372

Potassium-Phosphate Buffer [0.1 M] to 100 mL

1.5 Ringer's solution, pH adjusted to 3 with NaOH

Component	Amount (g)
NaCl [150mM]	8.76
KCl [4mM]	0.29
$MgCl_2$ [2mM]	0.19
$CaCl_2$ [2mM]	15
HEPES [2mM]	2.38

Distilled H_2O to 1 litre

1.6 Fly food for 250 mL flasks

Component	Amount
Water	1000 mL
Nutri-fly premix food (Genesee Scientific)	188 g
Propionic acid (Sigma)	5 mL
10% Nipagin in ethanol (Sigma)	5 mL

Appendix 2: Primer sequences

2.1 Primers used in PCR validation of P450 sequences (chapter 5)

Primer name	Sequence
Ob_CYP315A1_F1	TGCACGATGAATTGTACACGAAACG
Ob_CYP315A1_F2	TCGCGAAATGAAGAAAAGCTAGCG
Ob_CYP315A1_R1	ATCTTCGCGTTAATTTCAATTTTATGGATTCC
Ob_CYP315A1_R2	ATTACCGCCTAATCGAGTCATTTCCG
Ob_CYP4AV1_F1	CGTTATGGAAGGATCTTGGCTCACG
Ob_CYP4AV1_F2	ATATTATATGCACTCGGTCGACATCC
Ob_CYP4AV1_R1	TAACATTTCTCTTTAACTTTATTTGAATGCATAC
Ob_CYP4AV1_R2	TCGTGTCCCAAAATAGTAATAGGATGATAGAT
Ob_CYP6AS151_F1	CGGTTTGGAGGGATTCTCTT
Ob_CYP6AS151_F2	AGATTGCGAGAATATCCATTTCTGTTTAAG
Ob_CYP6AS151_R1	GGTCTAAATTTTTGTTATCCTCAGATTAAC
Ob_CYP6AS151_R2	CGAAACCAGCAGCAAAGAATACGAAGG
Ob_CYP6BE1_F1	CAATGTCGCTAAGTACTTGGTTCGTAC

Ob_CYP6BE1_F2	TGTCGGCGGCGTTTGGAATAAACGTC
Ob_CYP6BE1_R1	CTCAATCTCATCAGTCATTGCACCGGTT
Ob_CYP6BE1_R2	GTCTCTGTAGTTGACCGTGTCCTCGAAC
Ob_CYP4G11_F1	TAACCATGTCTACTGCTGGACCGGAA
Ob_CYP4G11_F2	TGGCTCTTCAATCTGACCAAGTACGG
Ob_CYP4G11_R1	ATTTTAGGCGGTTGCTGCTACGGGTT
Ob_CYP4G11_R2	CTTCTCACCAACGTCGTCGTCAACAT
Ob_CYP9BU2_F1	GGGACCGGTGTTAGCAATGA
Ob_CYP9BU2_R1	AAGATCATTGGAACG CCTAGGT
Ob_CYP9BU1_F1	AACTGAGTCCGAAGAGCCGA
Ob_CYP9BU1_R1	CCT AGG CTC AAC TCG CAA CA
Ob_CYP9R1_F1	CCACAGATCCTTTCAGAACGAC
Ob_CYP9R1_R1	TTCGAACCTCCCTCCATCTG
Ob_CYP9R38_F1	CCACAGATCCTTCCAGGACAAT
Ob_CYP9R38_R1	TCGTTCGAACATCTCTCCATCTA
Ob_CYP336A35_F1	AATGGCGTCTGCGTGTTTTACACTTG

Ob_CYP336A35_F2	TCCTACCAAGGGAGTTCGATCAGCTG
Ob_CYP336A35_R2	TGGATGTGTCGCCAGGTAATAGCTAAC
Ob_CYP307B1_F1	GAAGATGATCCCTTTGACAGCCACC
Ob_CYP307B1_F2	TCCTAATGCCCCTTCATCATCGTAAC
Ob_CYP307B1_R1	TACCGTCTCTCGAAACGGAACTTG
Ob_CYP307B1_R2	CACCGATTATGTCCTCAAGCACGAAC
Ob_CYP18A1_F1	TAATGTTCGTGGAACATGCGGCCCA
Ob_CYP18A1_F2	TCAAGAGGTTCATGGATTTGATCGAGG
Ob_CYP18A1_R2	ACGCGTCAACTAAATCTCGCACAGTAC
Ob_CYP6AS125_F1	AGATGACTGGCTTTGAACTTCTTTGTGG
Ob_CYP6AS125_F2	TGGAGACTAAAAGATGGCGACCGCTAAG
Ob_CYP6AS125_R1	ACGCTAGTACAGTTAGTTTAGACATTTTTAAT
Ob_CYP6AS125_R2	ATTTCACGGAACTTTGATTCCTTTTGC
Ob_CYP9DN1_F1	ACAATGGATCCTTTCACGTTAACTCTCT
Ob_CYP9DN1_F2	TGTATTGGCGAGAGATACGATGAGCTC
Ob_CYP9DN1_R1	TAATTTTCCAATTTTCCATTTGCTTCTTGC
Ob_CYP6AQ55_F1	CAAAATGGCTCTGATAACGCAAAATTGG
Ob_CYP6AQ55_F2	TCGATCTTCTGATTGAGCTGAAAAGG
Ob_CYP6AQ55_R1	TAACCGGCAACTGTTGTAAGCTTCC

Ob_CYP6AQ55_R2	AGATTGTTCAAGAGCGTTGTGAATCTC
Ob_CYP336L1_F1	ACATGGCTTTTGCATTCGTTATCC
Ob_CYP336L1_F2	TGGAAAGTTTCTTCAGGGACATTGTT
Ob_CYP336L1_R1	AAATTTTGGAAATATGAACCCAGAGTCC
Ob_CYP336L1_R2	AGTGAAACTAATTGCAACAGCGGATG
Ob_CYP6AS121_F1	TTTATAACTATGTCTTGGTCAATGTTTG
Ob_CYP6AS121_F2	TCATAAGAATCACCAAAGAAAGTATAGATT
Ob_CYP6AS121_R1	ATTTAAGCTTTTGTCATTTTTAAATATACTTCG
Ob_CYP6AS121_R2	ATAAAGCATGAGTGATCGTTACAGAGG
Ob_CYP305D1_F1	TGATGTTCATCACCGTGGTACTGATAATC
Ob_CYP305D1_F2	TGCCTGCTGTATTAAATGTCCTTTGG
Ob_CYP305D1_R1	TATCGTGGCATAACTAATGACTGGTAAGG
Ob_CYP305D1_R2	AGCTCGTTGTTCAGCGTTACAAGAAG
Ob_CYP4AB3_F1	AAATGATCGTTGTGATATTACTTCTTGTGT
Ob_CYP4AB3_F2	GAACGAAAAGAATATCACAATCAGACCG
Ob_CYP4AB3_R1	AACAGGTAGTCTCAATAGGAATAAATTTCGTC
Ob_CYP6AS127_F1	ACCAAAATGGCGACATATTTGGAAATT
Ob_CYP6AS127_F2	GAGAGAATCACTATAAGTACGATGGAGTAT
Ob_CYP6AS127_R1	TGTTGAAGATACTAACTTTCTAGCTTCGT

Ob_CYP6AS127_R2	AGTCAATTCATAAATAGCATTGGCTATCGT
Ob_CYP6AS136_F1	GAACATCAACGAAAATGCTGGGCTAC
Ob_CYP6AS136_F2	TGTACGAACTATTGGGTTACATTGTACC
Ob_CYP6AS136_R1	ACACTAACTTGTTAATTTTGTTATTTTCAGAT
Ob_CYP6AS136_R2	GCTCATTGTGGTCGAAGAAGTTTCAAAC
Ob_CYP6AS123_F1	TGGAACAACATGATAGAATATTTCCAAATAC
Ob_CYP6AS123_F2	TGAAATGGGCAGAAGATTTTTAGCAC
Ob_CYP6AS123_R1	GTATCAGTTTTACATTTTTGTTATCTTTAAGGTT
Ob_CYP6AS123_R2	AGCTTTTCAGGGTGTTCTCGAAGTTC
Ob_CYP301A1_F1	TCAATGAATCCCAGAATGAATTGCCAC
Ob_CYP301A1_F2	TCAGAACCTCAGAAGATCATCGACG
Ob_CYP301A1_R1	ATCTTGGCAGTAATTTGAATTTCAGCTCC
Ob_CYP301A1_R2	ATTAAAGACAGATCAGCTTCATTCACTG
Ob_CYP4AA1_F1	TATGGTTGTTACAGCCCAACAAATATGG
Ob_CYP4AA1_F2	TCGACTGACAAAACACGGAAGACAAG
Ob_CYP4AA1_R1	ATTTTTGATCCCGCACAACTACTTTCAC
Ob_CYP4AA1_R2	AGCAAGGATTCTTCTCGTTCATTTCAAC

2.2 Primers used to confirm successful recombination reactions of candidate genes into the baculovirus genome (chapter 6)

Primer name	Sequence
Polyhedrin- F	AAATGATAACCATCTCGC
V5- R	ACCGAGGAGAGGGTTAGGGAT

2.3 Primers used to produce transgenic *Drosophila* (chapter 6)

Primer name	Sequence	Application
Dm_CYP9BU1_F	CCAGAATTCATGGAGTACCTGACCATCACCCT	Amplification of sequence
Dm_CYP9BU1_R	ATAGCGGCCGCGGATCCAGACATGATAAGATA	Amplification of sequence
Dm_CYP9BU2_F	ATAGCGGCCGCGGATCCAAGCTTGCATGCCT	Amplification of sequence
Dm_CYP9BU2_R	TATGGTACCGGATCCAGACATGATAAGATAC	Amplification of sequence
Dm_CYP9R38_F	CTAGGTACCGGATCCAAGCTTGCATGCCTGC	Amplification of sequence

Dm_CYP9R38_R	TTATCTAGATTAGAAGGCCACGCCG	Amplification of sequence
Bt_spacer_1_F	GTCCGGTACCGGTCCAAGTTAATTTG	Amplification of sequence
Bt_spacer_1_R	CATAGGTACCTGAACAAATTGAAAATTGTCTGGCG	Amplification of sequence
Bt_spacer_2_F	ACTGCGGCCGCTTTCCGAAAGAAAGTGATTGGTGT	Amplification of sequence
Bt_spacer_2_R	GCGGCCGCGTGGATCTGCTTCAAATGTTGGAG	Amplification of sequence
Dm_polyA_R_seq	CTGCTCCCATTCATCAGTTCC	Sequencing
Dm_CYP9BU1_F_seq	CAGACCTGCTTCGTGTTCCAGATG	Sequencing
Dm_CYP9BU2_UAS_R_seq	CGCTTCAGCACGTTCAGGTTCTTC	Sequencing
Dm_CYP9BU2_polyA_F_seq	CCATCCACACCGATAGCAAG	Sequencing
Dm_UAS_F_seq	TAGCGAGCGCCGGAGTATAAATAG	Sequencing
Dm_CYP9R38_R_seq	CGCAGGTTGAACAGGTTCTTGCTG	Sequencing
Bt_spacer_1_F_seq	AGTTGCTTTCCGAAGAAGGAGTTG	Sequencing
Bt_spacer_1_R_seq	AACGAGGATGATAACAATCTGCAC	Sequencing
Bt_spacer_2_F_seq	AATAACCTTACTGGAGAATTGGCG	Sequencing

Bt_spacer_2_R_seq	TGTAATCCGTTGAATCGGCGATGC	Sequencing
Dm_CYP9BU1_F_qPCR	CATCCTGACCGCCATCTACT	qPCR
Dm_CYP9BU1_R_qPCR	GTGAACTCGTACATTCCGGC	qPCR
Dm_CYP9BU2_F_qPCR	TGCCGTGATGAAGATGGAGT	qPCR
Dm_CYP9BU1_R_qPCR	TATCGGGGTTCTCGTAGTGC	qPCR
Dm_CYP9R38_F_qPCR	CCTGCTGAACGTGAAGATCG	qPCR
Dm_CYP9R38_R_qPCR	AGCCGCCGAAGAAGAAGATA	qPCR
Dm_CYP9R1_F_qPCR	ATACCATCAAAGTGCGCGAC	qPCR
Dm_CYP9R1_R_qPCR	CTCCTCCTGCAGCTTCTTCT	qPCR
Dm_CYP9R39_F_qPCR	AAGAAGCCCTACGTCGTGAA	qPCR
Dm_CYP9R39_R_qPCR	CGAACACGTAGAACAGCAGG	qPCR

2.4 Primers used to determine the tissue-specific expression of candidate P450s (chapter 7)

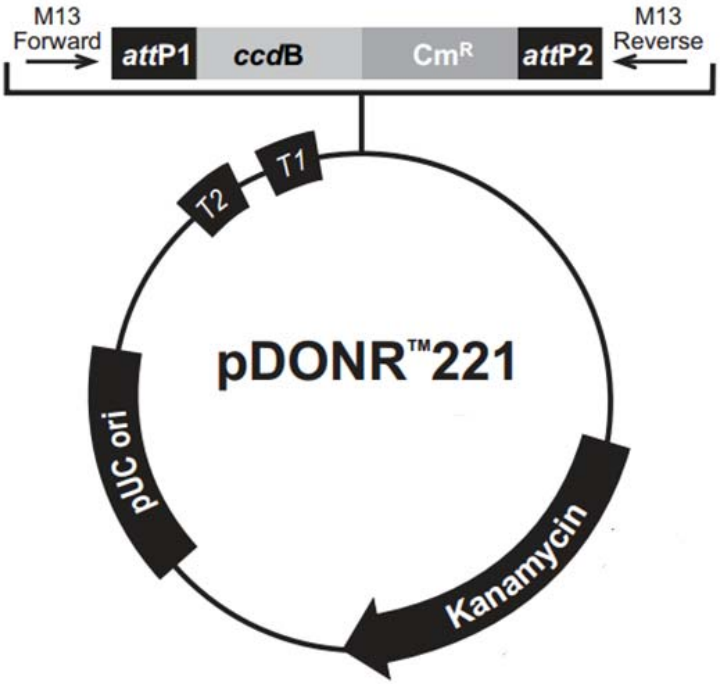
Primer name	**Sequence**
EF1_aF2	ACGGACAAACTCGTGAGCAT

EF1_aR2	TCGTGTTTCAGAGTAGGGCG
EF1_yF	CAAATGCTGGAACCTTGCCC
EF1_yR	CCCGGAAAACTTCAGAGCCT
Ob_CYP6AS151_qPCR_F1	CGCCGATAGGATGACAAAAC
Ob_CYP6AS151_qPCR_R1	GCCTTCCTTTGAGACCATTG
Ob_CYP336A36_qPCR_F1	TTCACCGATAGGGGTGTTTC
Ob_CYP336A36_qPCR_R1	TCGTCCGAATCCTCATCAAC
Ob_CYP336A35_qPCR_F1	CGGTTTGGAGGGATTCTCTT
Ob_CYP336A35_qPCR_R1	GGAGTCTCAGCCTCAGAAGG
Ob_CYP9DN1_qPCR_F1	ATGGAGGAGATCGAGGAGGT
Ob_CYP9DN1_qPCR_R1	TGTTACCCTAAAACCAGGCG
Ob_CYP6AQ55_qPCR_F1	TCCACGGCTGATAACGTACA
Ob_CYP6AQ55_qPCR_R1	TAGCGATCAACAGCATCAGG
Ob_CYP336L1_qPCR_F1	CGCCTCTGACGTGTTATCCT
Ob_CYP336L1_qPCR_R1	GCACCGTCGTATTTCTCGAT
Ob_CYP6AS127_qPCR_F1	TCGATGGGTTCTCCTTTCTG

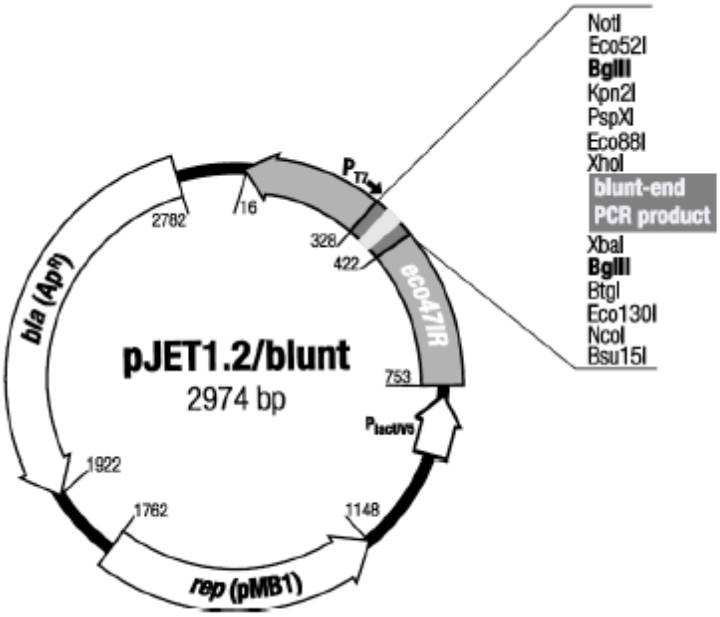
Ob_CYP6AS127_qPCR_R1	GCTCTGGAGGTCGAAAGATG
Ob_CYP9R39_qPCR_F1	TGAACTCTGGCACGTTTCTG
Ob_CYP9R39_qPCR_R1	TTCGGGAGTCATGGCTAATC
Ob_CYP9BU2_qPCR_F	GGGACCGGTGTTAGCAATGA
Ob_CYP9BU2_qPCR_R	AAGATCATTGGAACG CCTAGGT
Ob_CYP9BU1_qPCR_F	AACTGAGTCCGAAGAGCCGA
Ob_CYP9BU1_qPCR_R	CCT AGG CTC AAC TCG CAA CA
Ob_CYP9R1_qPCR_F	CCACAGATCCTTTCAGAACGAC
Ob_CYP9R1_qPCR_R	TTCGAACCTCCCTCCATCTG
Ob_CYP9R38_qPCR_F	CCACAGATCCTTCCAGGACAAT
Ob_CYP9R38_qPCR_R	TCGTTCGAACATCTCTCCATCTA

Appendix 3: Vector maps

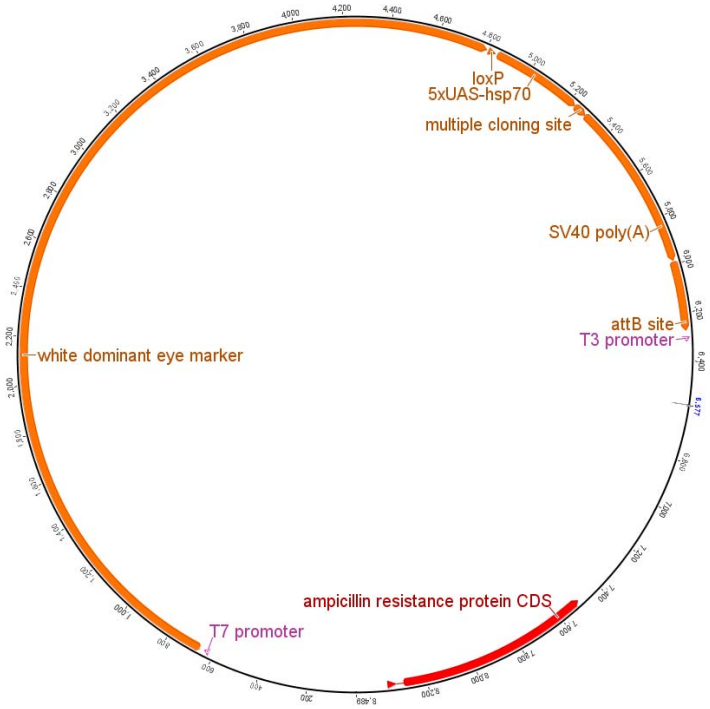
3.1 pDONR™ 221



3.2 pJET 1.2/blunt

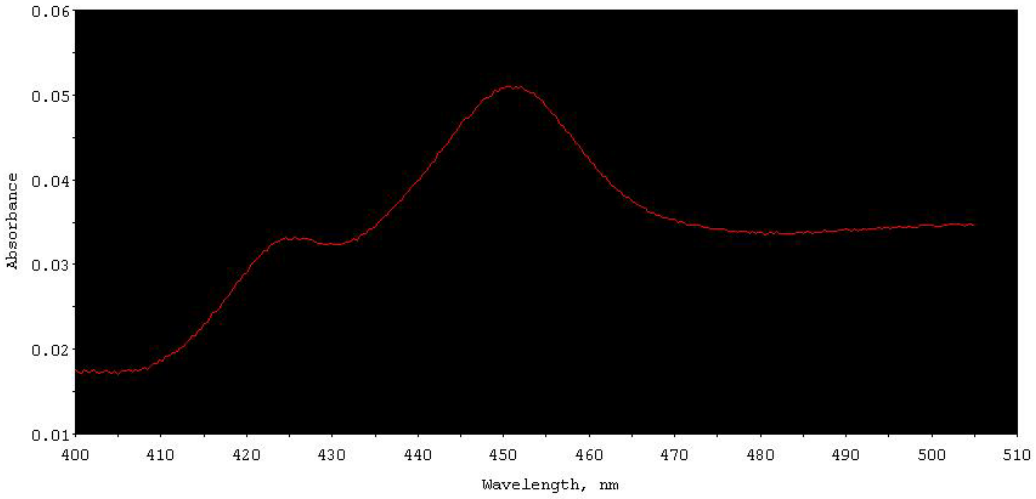


3.3 pUAST vector

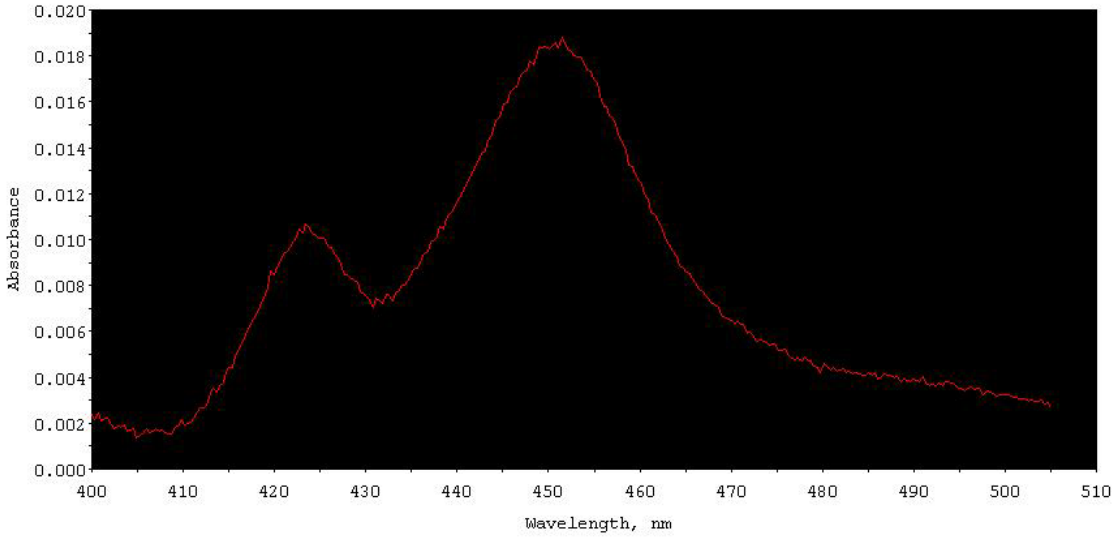


Appendix 4: CO difference spectra of recombinant P450s

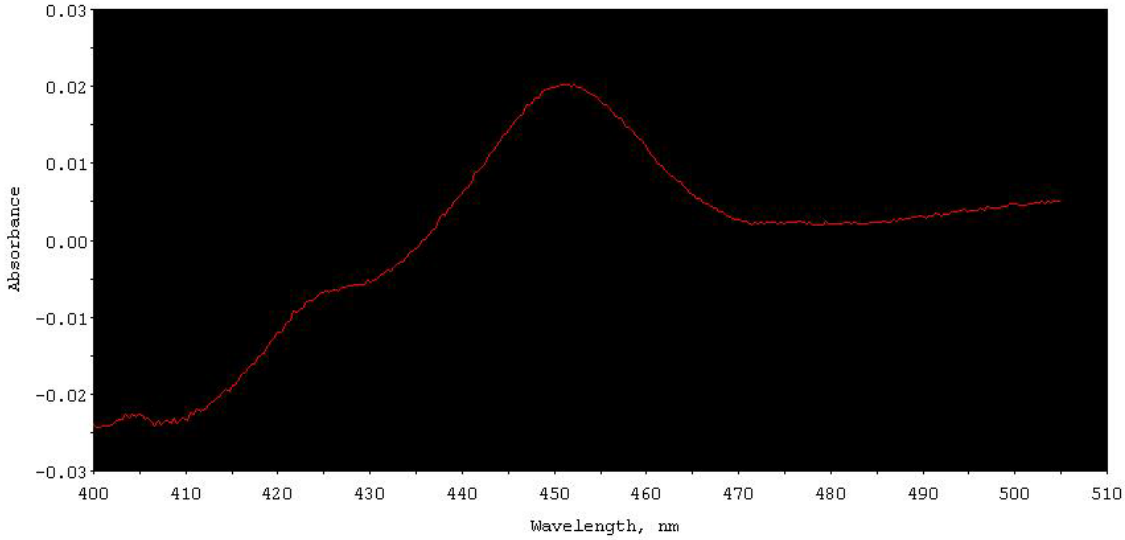
4.1 CYP9BU1



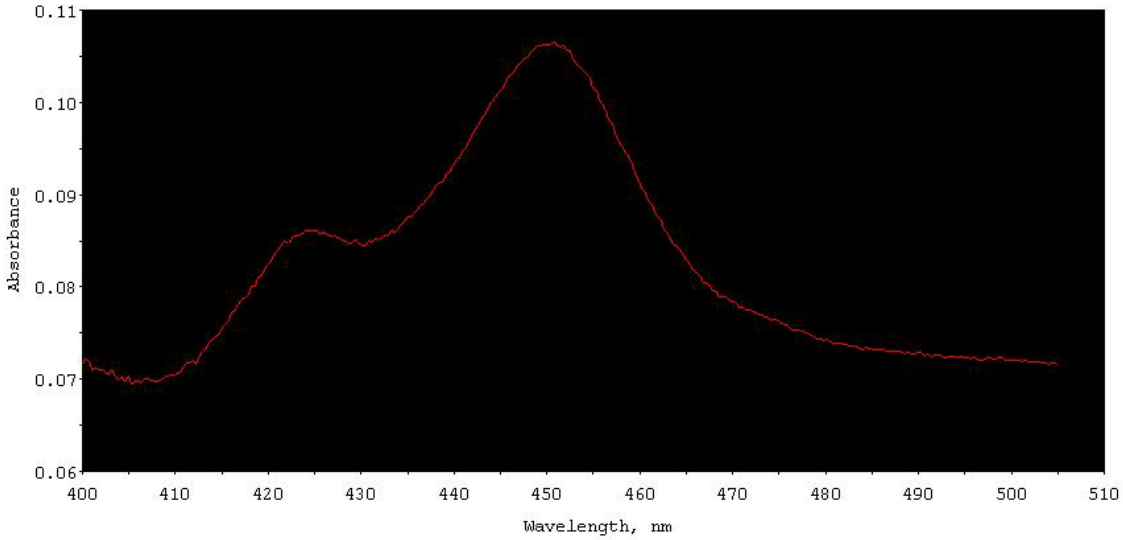
4.2 CYP9BU2



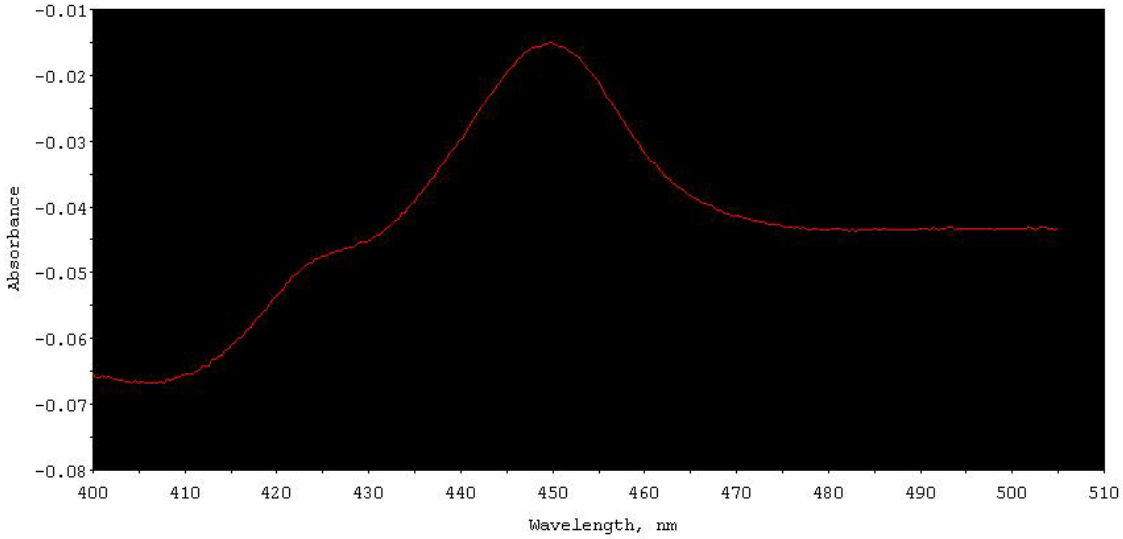
4.3 CYP9R1



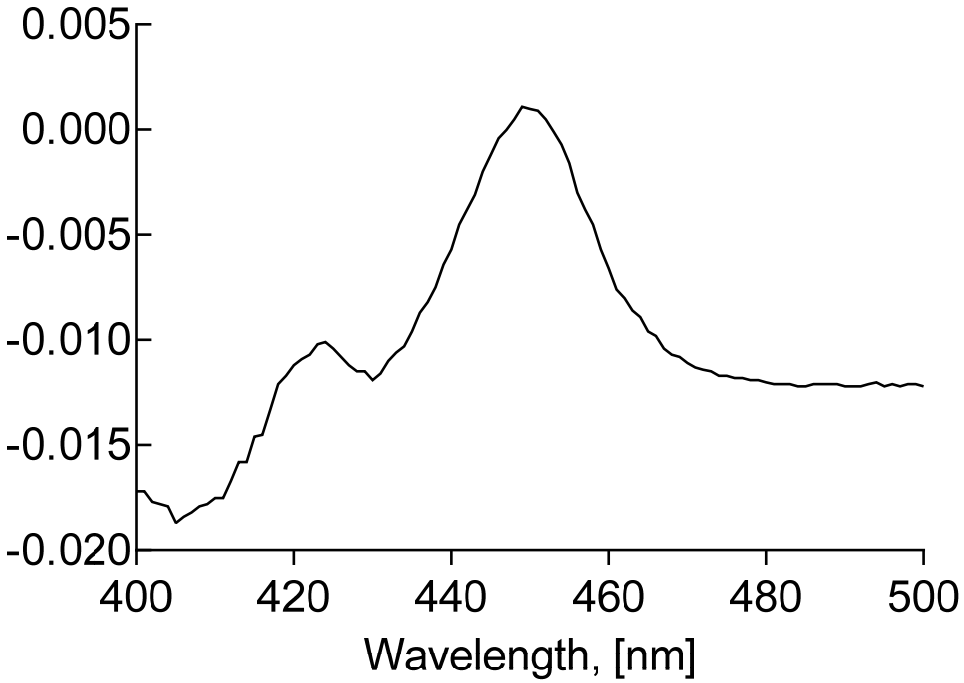
4.4 CYP9R38



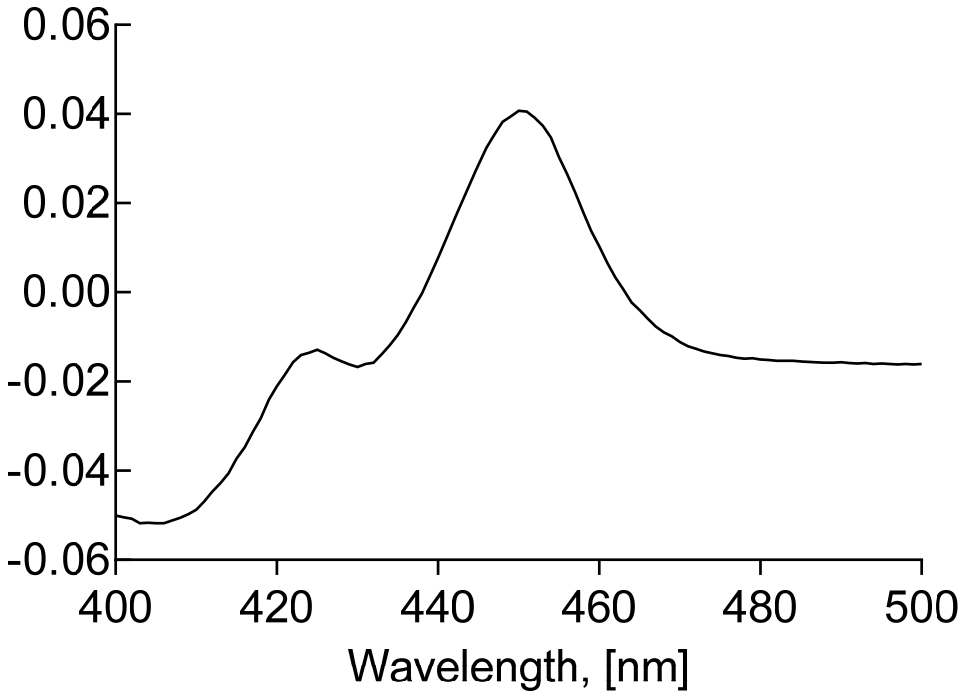
4.5 CYP9R39



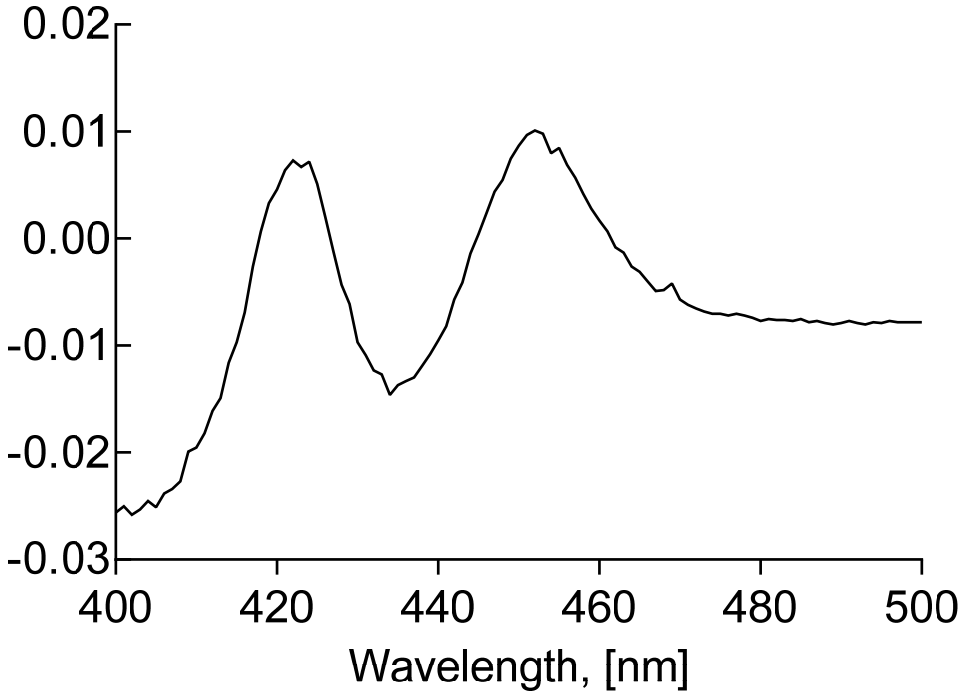
4.6 CYP9DN1



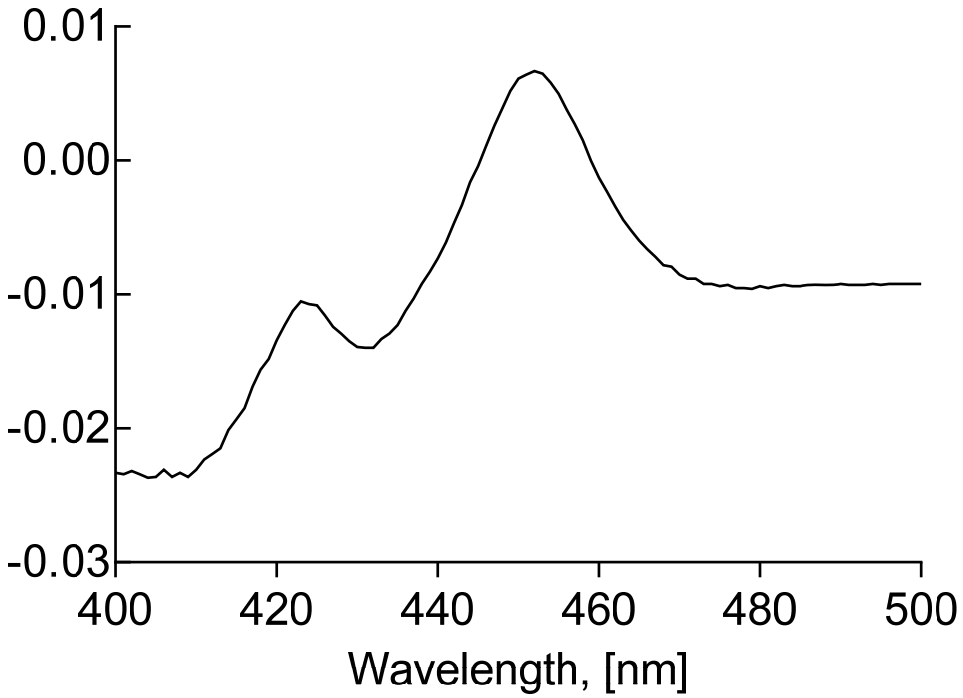
4.7 CYP6AS151



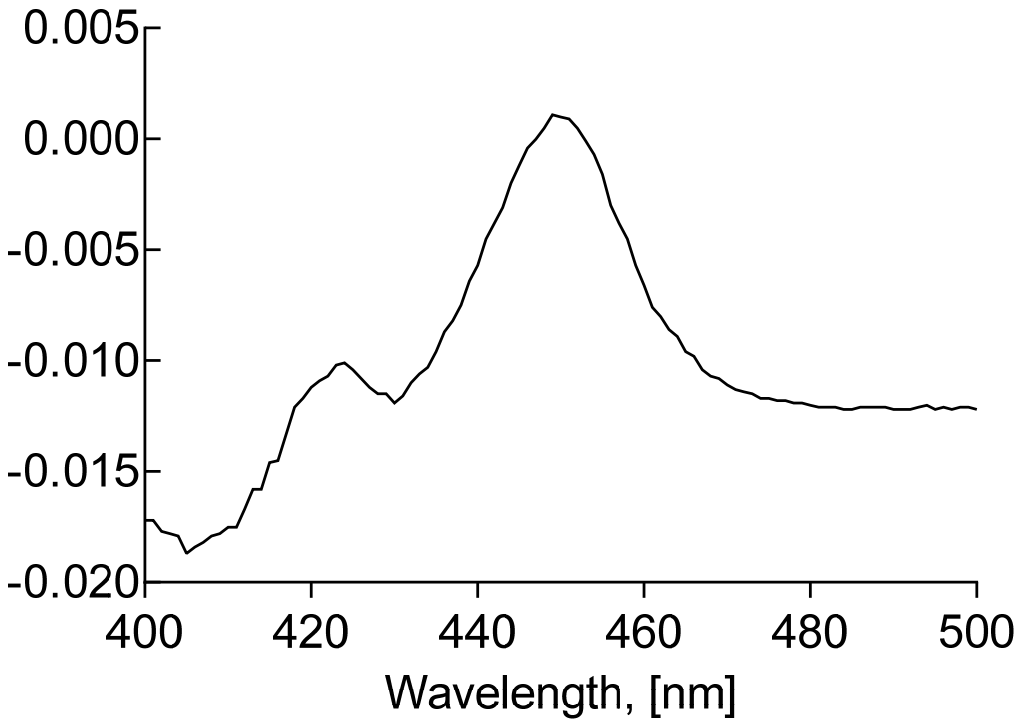
4.8 CYP6AS127



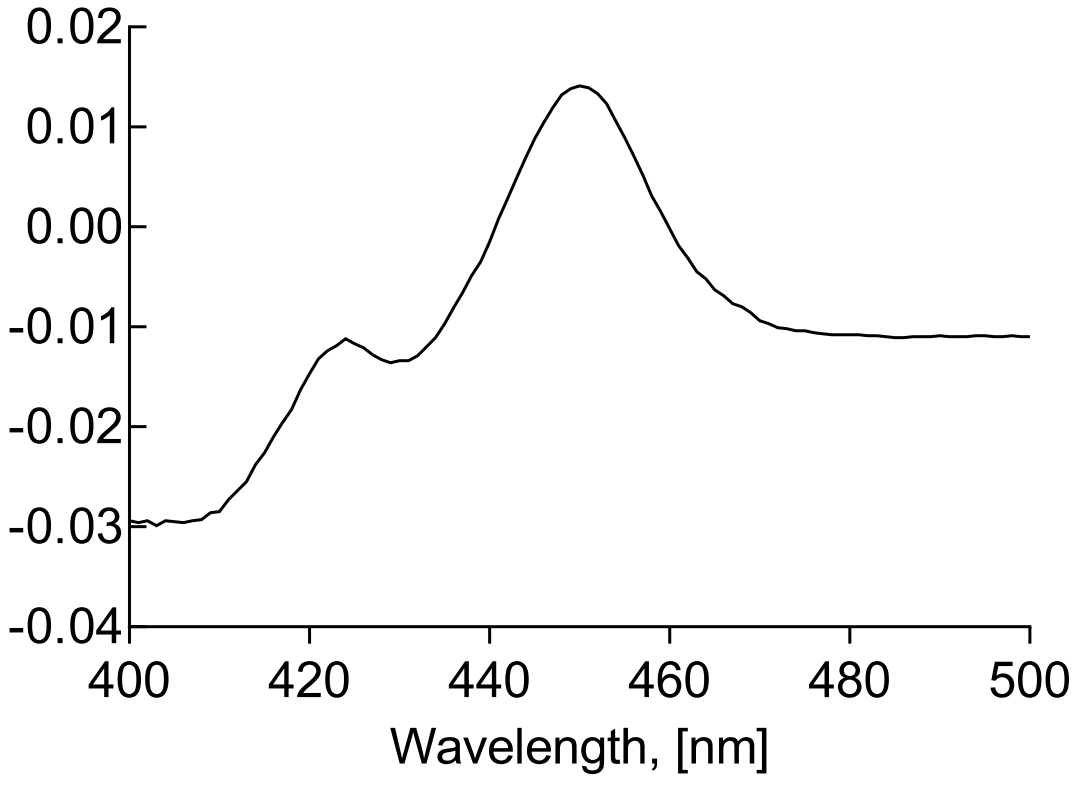
4.9 CYP6AQ55



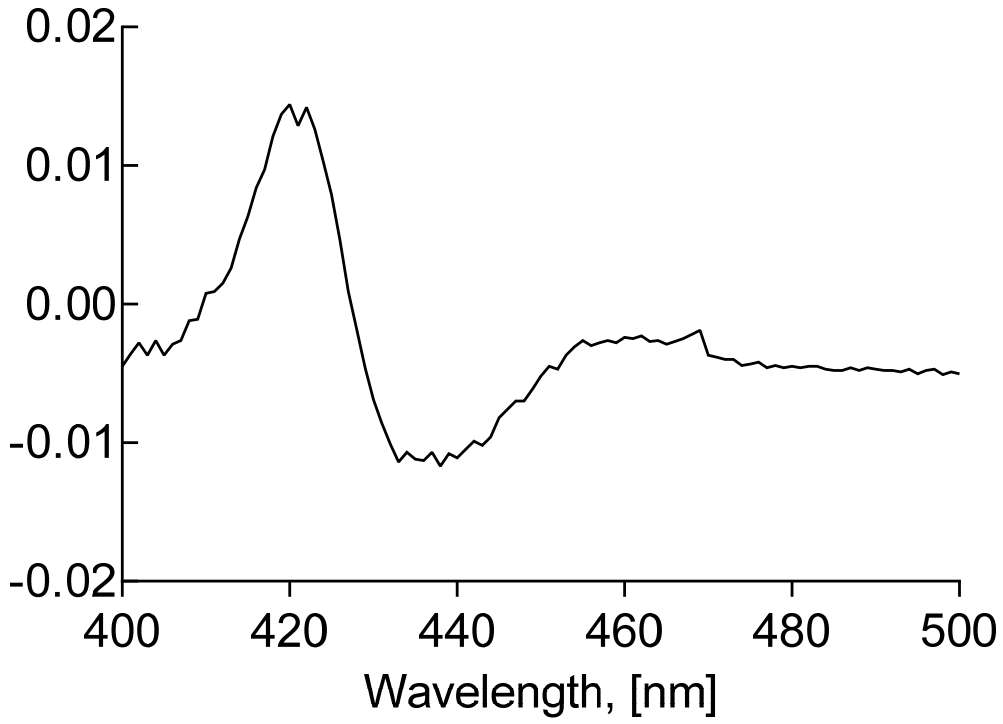
4.10 CYP336A35



4.11 CYP336A36



4.12 CYP336L1 (not successfully expressed)



Appendix 5

CYP name	Accession number	Length	Protein sequence
CYP4G11	MH500618	557	MSTAGPEIIAGSVAAASATGFSATTVFLSLLIPAVLLYYIYFRISRRHMIELGEKLPGPPALPIIGNALDLMGSSDTIF QNIYKRSFEYSDVIRLWVGPKLVVFLIDPRDVEVILSSHVYIDKSAEYRFFKPWLGNGLLISTGQTWRTHRKLIAPT FHLNVLKSFIDLFNANSRAVVEKMRKEGNKEFDCHNYMSELTVEILLETAMGVSKSTQRSGFEYAMAVMKMCDIL HLRHTKLWLRPDWLFNLTKYGKDQIKLLEVIHGLTKKVIQRKKEEYKSGKRNLIDTSSQKSDTKTTSVEGVSFGQS AGLKDDLDVDDDVGEKKRQAFLDLLIEAGENGVILNDREVKEQVDTIMFEGHDTTASGSSFFLAMMGCHPDIQEK VIQELDEIFGDSDRPATFQDTLQMKYLERCLLETLRMYPPVPIIAREINTDVKLASGDYTVPAGCTVVVATFKLHRQ PHVYPDPDTFNPDNFLPEKTANRHYYAFVPFSAGPRSCVGRKYAMLKLKIVLSTILRNFRVRSDMKESEFRLQAD IILKRAEGFKVRLESRKPVAATA
CYP4G202	MH500613	563	MEAVTMVTTYWQTAVFYSLIAISTTLLAVYLYIENLRVVRFGNKIPGPKTVPILGNALLTVGLHPNQVLDFLIQQDVY GPVVRAFLGTKLIVFMYHPRDCEIILNSSVHIDKAPEYRYFKQWLGDGLLISTGEKWRTHRKIIAPTFHLNVLKTFV PLFYQNSRDLVIRLRDQVGKEFDCHDYLSAVTVDILLDTAMGLRDTEKHKTGYDYAMAVMKMCDIIHRRQFNIPL RYDFLFNVSNMSREQEKLLGTIHGLTSKVIQRKKEEAYKSICSNIGQKQKKPNETESSQENKETSESNPNRKNEG SVRMHYVRDDLDDIDDNDVGEKKRLAFLDLMLELSRNGAGLTDEEIKEEVDTIMFEGHDTTAAGSSFVLCLLGIH QDIQDRVYEELEEIFKGSDRPCTFQDTVEMKYLERVILETLRLFPPVPAIARLLNEDVKIVTGNYVLPKGCTVLISPY RVHRLQEFYPNPDEFNPDNFLPERMQTRHYYAFIPFSAGPRSCVGRKYAMLKLKVLLSTILRNYKILSDLPEKDFR LKVDIILKRTDGFRVKIEPRNKSSQEVHV
CYP4AA1	MH500648	507	MVVTAQQIWDQTPGGVWTYLIILASVYILYILRTYIRTVIFVSRLNGPKTVPFLGNARCVLKDNLLHRLAHESQSYG RIVRIWLTGLPYVILIEPEDIQIVLSSMKHTRKIFFYKLLDNFLGKGLITRDVDTWKTHRKFLQPAFHLHILERFTSTF AECADHLMDQFLNKNNQEINITSFINDSVYDILSETVLGINRASRQSGLIMDDDLPFRKGQIMLLYRMVRPWLLIE WIYRLTKHGRQEEKQRKDLFDTCFKMMKEKRDLLRNKNINVNETKNTKRTSLLEYMVEMNEKNPCFTDEDIVEE CCTFMLAGQDSVGTATAMTIFLLANHPEWQEKCIEELDNIFNGSSRLPAMKDLKDMRCLEMCIKESLRLYPSVPII ARILGEDVKIGKHVVPAGCGVFISPYSTHRLPHHFPDPEAFKPERFNSENSEKRHPYAYIPFSAGPRNCIGNKFA MLEMKSMISAILRKCRLESIPGKKEVRPKFRMTIRAQGGLWVKVVVRDQK
CYP4AB3	MH500632	508	MIVVILLLVLLGVIVHFFILHYGKLGRMMNSIPGPQAIPIFGNLLTLQVSPHDLWKIMRKLSNQYYPIYRLWSFTIPFL NIRHPNDIETILGNPKSIQKSAVYDLLKPWFGTGLLTSSGRKWHLRRKILTPAFHFNVLRQFVDVFIEESERMIKML KSEQGPVVKDLMPIISEHTLNVICETSMGTSLKDKGVFQYKYRKAVYDMGQVQVYRLVRPWLHYEFLLACVPKG WEQCRLLKILHGFTKQIIRERKEYHNQTDGRYLSGFDDDSSGQKDMGIHKRRLAMLDLLIAAHRNNQIDDEGIRE EVDTFMFRGHDTTAMSICFTILLLAEHKEIQERARIEVNRVMEENEGKMTMSAFQNLSYLERCIKESLRLYPSVPFI

			SRAPEMDMKLSNYVVPADTVVHLHIADVHRDPKYWPNPDVFDPDRFLSENMHGRHPYSYVPFSAGPRNCIGQR FAMLELKATIGLLLHNFYLEPVDYLKDITITTDLVIRPAHPIRTKFIPIETTC
CYP4AV1	MH500612	514	MEGSWLTLILSMCLMTIILLLLVRRGKFLYTLRKVPYPPALPIIGNAYQLCCSPEEAFKKMIKWGQELGDIYLVWVG MRPFIFLYKAEAIQPLLSSSIHIDKSLEYKYLQPWLGSGLVTSTGEKWHFRRKLLTPTFHSGLLEVYLKTAIREAEV LISCLRKEIGKPAFDIVPYAKRATLDVICDSSMGCNVNAQTNFKNEYVQAINTLASISQRRFLNVWMSFDPIFKLTR WGQKYNHALGVTHEFVNRIIAERKAEWKAKKYRNCSEQSNKHQALLDLLLELSQDGKVLTDDDIRDEVNTFMFA GHDTTATSVSWILYALGRHPEYQEKVLNEYYEVTKTKQLTLDVLSKLTWLEACIKETWRVYPVAPLIARQIYHPITI LGHDIPIGSTVLVNSFLLHRDPRYFPEPDTYKPERFLPNGPKYPPYAFIPFSAGSRNCIGSKYAIMIVKILSLFILKNF HVISLDTEDQLRFSSELVLHNANGLRLKITPRKQELDVIILHSMHSNKVKEKC
CYP6AQ55	MH500625	515	MALITQNWGLDGLIVFSAIMVYIYFLMSRNFKYWAKRGVMEVPRTLLLGNFGDCLLGKRAPCEFLQDLYNQSKGL PYMGFYIFNKPYFLARDPELIKHIMVKDFNMFADRHSTADNVHDRLGYANLFMMKNPEWKAVRTKLTPIFTSGKL KKMFDLMLLIAKDLENCLQSMDLEGNGKVIELKDLCANVTTDMIGSTAFGLKVNSLENPDSPFREHGRQIFSYSLK RSLEFIIIFFLPEYVKYTRPKLFGKSTNEFLRNVFWDVINERIRSQEKRNDLIDLLIELKRKHENDTDLADFKFDGDD LVAQAIIFFTGGFETSSTTMSFTLYELAVDQEVQKTLRKEIHNALEQSGGKITYEMITTLPYLDMVISETLRKYPPLA FLDRVTTANYKVPNSDLVLPKGTPVYIPMMGIHRDPQYYPDPDKYDPSRFTEENKQKRPNFTYFPFGEGPHVCI GLRLGLMQSKLGVVQLIKDYEVTVCDKTTIPMTLNPRGLTTTAEHGIYVNCRKLTTVAG
CYP6AS121	MH500627	499	MSWSMFETVGFFVGILVLLYYYSTSVLEYWQKRGVKGPKPIPFLGNFTNVFLGRSSVNDCFVKAYYEFKDEPMV GVFGGHIPHLIIRDPDLIKDVLIKDFSTFVNRITVPNELEPLSVHLFGLEAKRWKPLRARLSPVFTSGKLKEMFGLLV ECGNHFEEYLKKLVEKGEYIATREMSAKYTTDVIGSCAFGIEMNALAAEDSEFRKMGRAIFQTNVKSVIKDRLREY PFLFKILGRFLLDSEVDDFFIRITKESIDYRIKHNFHRHDFIETLVDLKKNPGNISDKQLSDSFLAAQSFVFFAAGFET SSVTITHALYELAYNHSIQDTLRSEIKDVLQRSNGEITYDNIKEMKYLDAVLQETLRKYPVVLWLSRTAMTNYTFSG TKVTIPKGQHVVVPVDAIQNDPDIFPKPEIFDPNRFLDDNAKSRHPMFYLPFGDGPRNCIGARFAKIQSKVAMIKIL SNFKVDVCEKTITEYKRDIKSFVLLQPTHEVYLKMTKA
CYP6AS122	MH500639	497	MWSVLEIVGFLVAILILLYYYSTSILEYWKERGVKGPKPIPFLGNFKDVYLGKISMNDCFVKAYREFKGEPMVGVF SGHIPHLVLRDPDLIKDVLIKDFSNFVERMVVPNEVEPLADELFSIEAKRWKPLRARISPVFTSGKLKEMFNLLVEC GNQFEEYLKKLVEKDVYIECRDISAKFTIDVIGSCAFGIEANALAAEDSEFRKMGKAIVRTSVKSVIKDRLREYPFLF KIFGRFLLDSEVDDFFIRITKESIDYRIKHNFHRHDFIETLVNMRKEPGDISDKQLSDAFLAAQAFVFFTAGFETSSL TITHALYELAFNQSIQDTLRAEMKDVLKRNNGEITYDSISELKYLDAVFKETLRKYPVVLWLSRTAMTNYTFPGTKV TIPKGQHVVVPVDAIHNDPDIFPEPEVFDPNRFLDDNAKGRHPMFYIPFGDGPRNCIGARFAKIQTKVAMIKILSNF KVDACEKTPKYNRDPKSLMLLQPEHGIYLKMTKI
CYP6AS123	MH500644	499	MIEYFQILCGITIVLLYLYYHHTSNYDFWKKRNIPGPAPTILFGNLKEIAFKKESMSESVKRLYDEFKHEPLFGIFEAT

			TPTLIVNDLDLIKDVLVKDFPIFADRGLPYYKKVEPLAEHLFFLESERWKPLRAKLSPTFTSGKLKEMFPLIIDCAQH LEEYLDKMIEKQEPIECRELAAKFTTDVIGSCVFGIVTNALSDENSEFREMGRRFLAPCFRTTVRTAIRQFFPRLYV LIGNYIQAVGVSEFFINMVSDTMRYRKKNNIHRPDFINTLMELREHPEKLPTVELTDEFLTAQALVLFIAGFETSSTT ISHTLYELAQNHEIQDKLQKEIKQHDEKYGKTLTYDQIKGMKYLEKVFKETLRKYPILPMLPREALKNYTFNGTKITI PKGTKVWIPAFPIHRDPNIYPDPDRFDPERFTEEAVANRHPMSFLAFGDGPRICIGARFANYQVKLGIITILRNHKV DVCKETIIPFKQEPQALLLSLQGGITLKITKM
CYP6AS124	MH500646	501	MIDVFQTLLGIALVLVSVYIYNYYNEVTNYWKKYKIPGPKPHLLFGNIIQFIALKKSISECVKQLYDEYKEEPVFGIFD RTTPMLVVNDLDLIKDVLIKDFSLFTDRGLSFYPKVEPLTEHLFVLEPERWRPLRANLSPVFTSGKLKEMFLLMLD CSMYLEKHLAKIADKEEPVDCRELSAKFTTDVIGNCVFGIDTSALSNEDSEFRRIGRSVLAPSTRATIRNFVREILP FLYKTIGHLLQPPGADKFFTKMVVDTINYRKENNIYRPDFINILMELKNHPEKLQNVELTDTLLTAQAFVFFVAGFE TSSTTIANTFYELAQNHGIQDKLREEIRQSDTKYGKILTYDRVKEMKYLDKVFKETLRKYPIFPVLMRQALKNYTFR ETKITIPKGTKMWIPVYGIQRDPNVFSDPEKFDPERFSEEAVAARHSMSYLPFGDGPRNCIGARFAYFQSKVGIIT VLRNYKVDVCEKTIIPYKPDPRSFLLSLKGGVYLKITRA
CYP6AS125	MH500622	501	MTGFELLCGLVLIFLTFYYYLVCDYDFWKRRGISGPKPVPLFGNFMNSLLGKISIGDEMVKYYKQYKHLPMVGLYI RKQRVLGVIDPDIIKTILIKDFSKFADRGLKVNKVAEPLSQHLFSLETKRWRPLRTQLSPIFTSGKLKDMFSLILECS VNFEKYLDTLVAEGKPIDCRELTARFTTDVIGSCSFGIDANAMSQKESKFREIGRQIFYPGWKQILRMRLRESLPK LYTLIGYILPHDEMTKFFTEIVLDTIEYRKKNNIVRHDFINKLMELQAHSEKLGDIELTDTLLAAQAFVFFAAGFETSA ATIANALYELALNQELQVKVREEIRERYALNNGEWPYEDINDMPLLDAVFKETLRKYPPITVIMRKAEENYTFEDLK LTIPKDTRIFIPSYAIQHDPEIYPDPEVFDINRFTADAVAKRHPMHYIPFGDGPRNCIGARFAHFQTKIGLIKVLLKYK VDTCKETRPYEIEPRSFLLAPKNTIILKMSKLTVLA
CYP6AS126	MH500614	501	MATYLEISCGFIAVFLALYYYFTSTFNYWKKRGIKGPEPIPVFGNIMAPMLAKQSLADFLTNLYKKYKNEPMIGIFA RREPILIILGPDIIKDVLIKDFSKFANRGFKIHEIAEPLSQHLFFLEVERWRPLRTQLSPVFTSSKLKGIFSLILDCAKH LEKYMDTLVQKGEPIEIREVAAQYTTDVIGSCAFGIEMNSMSESESEFRKIGREIFSTNLTTFLKFKMKEWMPQLY NLLGYVLPKDKTMAFIERITISTMKYRKHNNIVRSDFINTLLELQKHPERVSGIQLTDTLLAAQAFVFFAAGFETSST TIANAIYELAQNQEIQDKLREEIKEFDAKNNGEWKYETVKQMKYLDKVFRETLRKYPPLSFLSRKATENYTFENPK LTVLKDAKVWIPLFSIHRDPEVYPYPDKFDPERFSEDAVKVRHPMHYLPFGDGPRNCIGARFAIYQSKIGIITILRNF KCDILEKTEIPYEFDPIAFVMCPKNGLYIKITNLES
CYP6AS127	MH500637	501	MATYLEISCGLVAVFLALYYYFTSTFNYWKKRGIRGPKPIPVFGNIMAPMLAKESLANFLTNLYTKYKNEPMIGIFV RREPILIILDPDIIKDVLIKDFSKFANRGFIRHEIAEPLSQHLFALEVERWRPLRTQLSPVFTSGKLKGIFSLILDCAKH LEKYMDTLVQKGEPIEIREVAAQYTTDAIGSCAFGIEMNSMSETESEFRKRGREIFATNFTALLKFRMKECMPQLY NLLGYVLPKDKTMAFIERITISTMEYRKHNNIVKSDFINTLLELQKHPERVSGIQLTDTLLAAQAFVFFAAGFETSST TIANAIYELTLNQKIQNKLREEIKEFDAKNNGEWKYETVKQMKYLDKVFRETLRKYPPLPFLSRETTESYTFENPKL

			TILKDAKVWIPLFAIHRDPEVYPDPDKFDPERFSEDAVKVKHPMHYIPFGDGPRNCIGARFAIYQSKIGIITILRNFK CDISEKTEIPYEFDPVAFIMCPKNGLYIKITKLES
CYP6AS128	MH500640	435	MADYFQLLSVFAVLFLAVYYYFTSTFDFWKNRGIPGPRPIPVFGNVKDVILGKYAMNEYILKLYKEYKNEPMIGIFK NRSPSLVLLDLDLVKDVLIKDFSTFNDRGHMVFERTEPLSLNIVSLEAKRWRPLRARLSSVFTSGKLKDMFPLILE CSNHLEECLEKIVEKDGLLDCREIAARFTTDAIGSCAFGINMNALSDEGSKFRQIGKKMFKPDIKKRLLGTVRETM PWLYNLLGFVIPRNEVTTFLTNIVSEMIKYRKENNIVRPDFINVLMDLKDNPNKLENIKVTDSLLTAQAAVFFAAGFE TSSTTVGHTLYEMALNPDIQDKLRQEIKEVYTKNKGNWTYDSIRDMKYLDKVFKETLRKYPPAGLLTRRCNSNYT FNGTKVSIPTDTEVFVSIHAIHTDPNIYPDPDVFDPERFNEDAEAARHSMSFI
CYP6AS129	MH500642	499	MADYFQLLSAFAVVFLALYYYFTSTFDFWKNRGVNGPQPVPVFGNIKDIIMGKCALGTYTVKLYDEYKNEPMVGI FVRRSPHLVLIDLDRIKDVLIKDFSTFSNRATLIFEKTEPLSAHLFNLETERWRPLRTRLSPVFTSGKIKDMFPLILEC SNQLKECLEKIVEKDGLLDCREIAARFTTDVIGSCAFGISMNALSDEESEFRRMGKQIFKSNLTTILKGSFKEAMPK LYNTLGFVLPQTEITKFLTTIVSETIKYRKEHNIIRPDFINLLIDLKDNPHKLENIELTDTLLAAQAFVFFVAGFETSSTT IGHALYEMALNHDIQDKLRQEINEFYAKNKGNWTYDDVKGMSYLDKVFKETLRKYPPGLLLRRKSTNNYTFSGTK VSIPKDTGVLIPVYAIHKDPNIYPDPEVYDPERFNEDAVAARHPMAFLPFGDGPRNCIGARFAVYQTKIGLIQMLTN FKVDVCDETLIPYVQHPTSRVFAPIRSIILKISKV
CYP6AS130	MH500641	501	MAIYLEIFCGIAALCLAFYYYFITVLDFWKVRGVPGPQPIPVLGNIKDVMLMRMSMSDYLKKVYDEYKHEPMVGIF TRKTPILLLHDPELIKDILIKDFSKFADRGLPVYEKAEPLSPHIFNLEAKRWRPLRSRLSPVFTSGKIKDMFPLILECA DHLEQYLEKVVARGEPVECRELTAKYTTDVIGSCAFGIEINAISDEESEFRRVGRTVFALNWKQLLRFRARQMFP RLYNWLGYILPPSEITTFFTKVIVDTINYRENNNVTRPDFVNMLMELKKHPDKLENIKLTDSLLTAQAFVFFVAGFE TSSTAISNALYELALNPEVQIKLRQEIREFYDKNDGKLKYDEIKDMTYLDLVFKETLRKYPPGPLLLRKSICNYTFEG TKVTIPKKTFVWVPIYPIHRDPDIYPNPDAFIPERFTEEAVASRHPMHYLPFGDGPRNCIGARFAVYQSKVGLIKIL RKYKIDVCEKTMIPYQIDPAAFLLAPKGGIYLKITKVES
CYP6AS131	MH500655	501	MGNYFEILLSIAVILIAFYYYAISNFDFWKKRGVVGPTPTPFFGNTMDLIFTNISLRHYIKTAYDQYKNEPMIGLYLM KKPSLILNDPELIKHVLIRDFSKFADRGALVYEKTEPLSPHLFNLEAERWRPLRARLSPIFTPGKMKEMFYLIIECSQ HLEKYLAKEVEKGEPLECRELTTRFTIDVIGSCAFGIEMNALADGRSEFHQKGRNVFDATFENVFRFKMKQFFPE LYDLLGYIIPERRLASFFTKAVMDTIKYRQENDIFRPDFINMLMELKAHPEKLNNIELTDSLLTAQAFVFFSAGFETS STTISNTLYELAVNQEIQNKLREEIMEYCSKDKGELEYETVKEMEYLDKVFKETLRMYPAVSLLIRNAISNYTFDGT KVSIPKGTKIWIPVFALHRNSDIYPNPDSFDPERFNEDAVAARNSMHYLPFGDGPRNCVGARFAIYQTKVGLIAIL RNFKVDVCEKTRIPYEFDPAAFLLAPKEGIYLKFTKIQS
CYP6AS132	MH500653	502	MASYFEILLSITVILIACYYYAVSNFDFWKKRGVVGPTPIPFFGNTKDIMFAKTSIRHYIKTTYDQYKNEPMIGFYLM KEPSLILNDPELIKHVLIRDFSNFADRGALVHEKTEPLSPHLFNLEAERWRPLRKRLSPVFTSGKLKEMFYLIIECS QHLEKYLAKEVEKGEPLECRELTARFTTDVIGSCVFGIEMNALADERSEFRQMGRDFFDTSSFESVSRNKMKEL

			FPKLYDFLGYIIPERRLSSSFTKAVTDIIKYRQENDIVRPDFINMLMDLKTHPEDLNNIELTDFLLTAQAFLLFLTGFE TSSTTISNALYELAVNEDIQNKLREEIMEYCSKDKGELKYETVKEMEYLDKVFKETLRMYPPGSLLFRNATSNYTF DGTTVSIPKGTTIWIPVFGLQRNSDIYPNPDSFDPERFNEDAVAARHPMHYLPFGDGPRKCFGPRFADYQTKIGLI AILRNYKVDVCEKTSIPYEFDPAAFLLTPKGGIYLKFTKVQS
CYP6AS133	MH500610	502	MASYFEILLSITVILIAFYYYAVSNFDFWKKRGVVGPTPIPFFGNTKDLIFANISLRHYIQTTYDQYKNEPMIGFYLMR KPSLILNDPELIKHVLIRDFSNFADRGALVHEKTEPLSTHLFNLEAERWRPLRTRLSPIFTSGKLKEMFYLIVECSQ HLEKYLAKEVEKGKPLECRELTARFTTDVIGSCAFGIEMNALADERSEFRQKGRDFFDTSSFENVFRNKMRQLFP KLYDLLGYIIPERRLSSFFTKAVTDTIKYRQENDIVRPDFINMLMELKAHPEKLNNIELTDSLLTAQAFVFFLAGFET SSTTISNALYELAVNQDIQNKLREEIMEYCSKDKGELKYETVKEMEYLDKVFKETLRMYPPGSLLFRNAISNYTFD GTKVSIPKGTTIWIPVFALQRNSDIYPNPDSFDPERFNEDAVAARHPMHYLPFGDGPRNCIGARFAIYQTKIGLIAIL RNYKVDVCEKTSIPYEFDPAAFLLAPKEGIYLKFTKVQS
CYP6AS134	MH500654	502	MASYFEILLSITVILIAFYYYAVSNFDFWKKRGVVGPTPIPFFGNTMDLIFTNISLRHYIQTTYDQYKNEPMIGFYLM RKPSLILNDPELIKDVLIRDFSNFADRGALVHEKVEPLSTHLFNLEAERWRPLRARLSPIFTSGKLKEMFYLIVECS QHLEKYLATEVEKGKPLECRELTARFMTDVIGSCAFGIEMNALADERSEFRQKGRDVFDASSFENIFRNKMRAFF PKLYDLLGDIIPERRFSSFFTKAVTDTIKYRQENDIVRPDFINMLMELKAHPEKLNNIELTNSLLTAQAFVFFLAGFE TSSTTISNALYELAVNQDIQNKLREEIMEYCSKDKGELKYETVKEMEYLDKVFKETLRMYPPGSLLFRNAISNYTF DGTKVSIPKGTTIWIPVFALQRNSDIYPNPDSFDPERFNEDAVAVRHPMHYLPFGDGPRNCIGARFAIYQTKIGLIA ILRNYKVDVCEKTSIPYEFDPAAFLLAPKKGIYLKFTKVQS
CYP6AS135	MH500611	502	MASYFEILLSIAVILIAFYYYAVSNFDFWKKRGVVGPTPIPFFGNTKDLIFAKTTIRHYIETTYVQYKNEPMIGFYLMS KPSLILNDPELIKDVLIRDFSQFADRGNPVYEKTEPLSPHLFSLEPERWRPLRTRLSPIFTSGKLKEMFYLIIECSQH LEKYLAKEVEKGEPLECRELAARFTTDVIGSCAFGIEMNALADEKSEFRQKGRELFDVSSFENIFRNKMRQFFPK LYALLGNIIPDRRFAPFFTKVITDTMKYRQENDIVRPDFINMLMELKAHPEKLNNIELTDSLLTAQAFVFFVAGFETS SATISNALYELAVNQEIQNKLREEIIEYCSKDKGELEYETVKEMEYLDKVFKETLRMYPPLTVLFRNVSSNYTFDGT KVSIPKGTKIWIPVFALHRNSDIYPNPDSFDPERFNEDAVAARHPMHYLPFGDGPRNCIGARFAIYQTKIGLITILRN YKVDVCEKTSIPYEFHPAAFLLTPKEGIYLKFTKLQS
CYP6AS136	MH500643	501	MLGYFEILCGIAALLFAFYYYSTANFNYWKDRGVVGPKPLPFFGNTKDLMFPIVSVPEFSRDIYLKYKGERMVGLF MQRSPVIFLKDPELIKDVLIKDFSKFSDRGFKVYEKTEPLSQHLFNLETKRWRPLRSKLSPMFTSGKLRDMFGLIL ECSEHFKQYLDKVVAKGEPIDIPDLTGKFTTDVIGSCAFGIDTNSLADEESEFRKVGKQIFTPTFGNVLRLKTSIYLP KLYELLGYIVPDKKFAPFFTKVVIDTMMYRKEHNIHRPDFINMLMELKDHPQKIDDIKLTDYLITAQAFVFFAAGFET SSTTMSNALYELAQNHDIQDRLRKEIEEHFENSDGNLKYEHIKEMEYLDKVFKETLRKYPPGALLPRTATSDYTFD GTKASISKGMLVWIPIFAIHRDPEIYPNPEVFDPERFNEDAVDARHPMSYLPFGDGPRNCIGARFAVYQTKLGLITI LRNYKVDICEKTIIPYHFAPGAFILTPKEGIYLKITKLTS

CYP6AS151	MH500615	504	MSWPLFEAAGLLAAILFLLYYYSTSLLQYWEKRGAKGPKPIPFLGNFKDMFLGKSSFNDCIIKAYYEFKDEPLIGVF SGHIPILIVRDPDLMKDVLIKDFSKFADRMTKPNEEIEPFSLHLFRLDGKRWKPLRTRFSPVFSSGKLKEMFYLILE CGNHFEKYLDTMVSKEGNKGAIIDCRDISAKFTTDVIGSCAFGIEMNALAAEDSEFREMGKRVFQTSWKTVLRDR LREYPFLFKIFARFILDYEIVDFFTRITKESIDYRIKHNVHRHDFIDVLVDLKQNPGKIELEDFNDLFLTAQAFVFFAA GFETSSVTITNALYELALNPSIQEKVRTEIQNVLKRNNGEITYDSIKEMKYLDAVFQETLRKYPVVLWLSRKATTNY TFSGTKVNIEKGQLVILPVFAIQRDPDIFPDPEVFDPNRFTDESAKTRHPMLFLPFGDGPRNCIGARFAKIQSKIAMI KFLSNFKVDVCEQTVKTYEIEKKSLLLLQPSHEVNLRITKI
CYP6BC1	MH500635	508	MWSIIRNFLEQFLLLGLFLSILYCFLTSTFNFWEIRGVPFRKPTVLFGNYASLLLFQISLPEGIKEMYEWFKDERFFG AFRVKSPVLILRDPDLIKNVCVKNFACFSNRGIPVNSQDPLSAHLFNLEGKKWKSLRSKLTPAFSSGKLKRMFYLL AECGEEFQKLIGSSSEADRPIEIRELAAKFTIDVIGSCAFGIQINALTDEESEFHRAAKKLSRPSYKATLWRMLRTA MPRVYKFLSVQVINPEVTRFFKNVVSQMLKQREEHGIKRHDFMDLLIELKNKGTLENEASNGHVSNDEDADTVE EIELDENSIAAQAFVFFAAGYETSSNTIAFCLYELALNAEIQERTRRDIQDAIDNRDGKLTYDAVQDMKYLDMVIAE TLRKYPPASLLSRRCEYQYQIPGSKVELPPGMRVIIPIYGLHHDPDYYPNPATFDPERFTEENKRTRHPYTYLPFG EGPRNCIGMRFALLQIKVGIISFLRKHRVEVCEKTVIPIKFSRRSLVTTS
CYP6BD1	MH500651	503	MAVLTSFLLNFGFTVVALLAILVLYVKYKRSYWKSRGVTSVPGHWLFGNVKEAALQKKSFANVFGELYQQASEK DVVLGFYIFHKPFLLVKSPELIKQILIKDFNIFPNRHFSAGSFHDEIGNTNLFTIQNPPWKHLRTKLSPIFTSLKLKKLF HLIVENSESMNKYLEDQFSNGTKTKSILLRDVTLRYTTDIISNIAFGVQVNSFNPDNTELFEKVQEGLNFSIKRGIQF ATMFFFPSVAPFVGAQMLGSSTDYFRKVFWDSMDARELNKVKRGDLIDLLVELKNEKQDNEDFKFTGDALVSQA AIFFIAAREASVTTMCFTLAELAKHPEIQKRTRAEILEKLAEHGMTYEGVRSMKYLYQVISETLRLYPPAPILDRVPI QNYKIPGSNIVIEKGTPVYITLTGLQRDPKYYRDPLTYNPDRYNEENKDEISQCTYIPFGDGPRACVGTRLGQLQS AIGILTIIKDYEVSFDSTCNCEIDNRNVFLSPVDKFNLKLTKL
CYP6BE1	MH500616	514	MSLSTWFVPEIVSVVIFGTIALYVYYKFFIFNFWRKKGVFYMEPTFPTGNITALIFGTKSQADYVKDIYDRNKKHRA FGIYMFQKPFLVVNDPSLIRIVLTKDFMNFHDRGVFCNEKTDPLSGQLFQLPGKKWRTLRVKLTPTFTSGKIKTMF FILKESADRLGKFLDEQGKIRGIIDVKDVYGKYSIDIIMSAAFGINVNSFRDPNNEFNYWGKKVFEPNIFWNAIVIWA PHILDKLSMPFTDRGVSNFFIKMFEDTVNYRDANNIERKDFLNLLMQLMKNGYVDVDEDSDEKDVTKKTETKLTM LEAAAQAYVFYLAGFETSSTTVTFCLYELAKHQDMQDKLREEIRTVIQKHGELTYDAVNEMTYLHKVISETLRKYP PLVMLNRICTEEIQIETTDIRIPTGTSVMIPVYGLHRDPDIYPDPDKFDPERFSEENIKTRHPYAYLPFGEGPRICIGL RFGLIQSKVAIISSLLRSKFKLAPNTPTTLEMKEGSLVLMAKGGVHLTIEPVQ
CYP9DN1	MH500623	511	MDPFTLTLFLTMGFLLLYHFLWQPMNYFKERGIKYESPVPILGNMASVVLRRTSMAEHFQRLYQRFSDVKYFGLF NFTNPVIVIRDPDLIASITVKNFDHFCNRRGFDIGDLDPLIGKNLSALRDDEWREMRRLLSPSFTSMKMKIMYHLIR DCADDFSDFIANQSKHGKVFDIKDIFGRYTTDVIATCSFGISIDSMRNPNNEFYVLARDTMSSQSSLSWKLMLGM

			CCPALCRTFGIRIFSEKVHRYFLNVVRETVKMREEQGITRPDMIQLMMDTKDKERSLTIEEMTNQAFVFFLAGYDT SSTFLSFLMHEIATHPEVKAKLMEEIEEVVRKTNGNPTYDALKNMPYMDAVMNETLRLDSIATSLDRVCVKEFQL EPASSGAEPVTLKPGDVVWFLPFSLQRDSKYVKNPTKFDPDRFLGKDAPPPSVNIPFGIGPRFCIGNRFALLESKI LMFYLLWRCDVEPCEKTQIPMKFSKHNIALTAENGFWLKFRARDKVCLQEANGKLEN
CYP9P2	MH500628	514	MESVSFSFPLKLLVLAFLIISLAKLISIVYIQHTYWKKRNAPYIKGVPVFGSSWRLFLRRISFPDCCKFIYNYYPNLRY VCVMDFSTPGVVIRDPVLIKEIAIKNFEHFPDHQSFINEKIDPIFGKNVFSLRGERWKEMRSTLSPSFTASKMRFIF ELVSKCSQEFVNYLYNHPEFSSSMEAKDAFTRYTNDTIATVAFGISVNSLRDRENEFYKKGADATNFGGLFRLMK FLLFRMNPRLTRMAGFSFLSRDTASFFKRVVFETVKARDEQNIVRPDMIHLLMEARDKEKLVSRQMTIDDITAQAF IFFLAGFDTSSTLMCYIVHELAINPDIQEKLRQEIDRYMQEGNGVISYEALSRMKYMEMVTSEALRKYPPLVLIDRL CARKFQLPPAGPGYNGVTVHPDNVVLFPVYALHHDPKCFPEPEKFDPERFSDENKDKIDPYTYLPFGLGPRKCI GNRFALMETKILIAHLLHRFYLKCTEKTKIQVEFSKKNFSITPDGGFWIGLEKRIV
CYP9P22	MH500630	392	MDPIFGKNVFSLKGDRWREMRNTLSPSFTTNKMKFIFDLVSKCSQEFVNYLYDHPEFSSMIEVKDAFTRYTNDVI ATVAFGIDVNSLKHRDNEFYTHGKDATNFSGVFRLLKFLLFRISPRMTRMVGIKFLSNSTSEFFWRVINETVKTRD ERGIVRQDMIHLLMQARDDEKHQITIEDIVAQAFIFFLAGFDTSSTAMCHAIYLLALNQDVQARLRDEVDRYLEEEN GKISYDSLSKMKYMDMVISETLRLYPPSLLTDRTCVKKLELPPARDGYDGMTINPGENVWIPIFAIHRDPKYFPDP EKFDPERFSDENKNNIIPYTYIPFGSGPRQCIGNRFALMEVKLLIVHLLQKFVIKPNEKTSIPLVFKKSSFTLIPKDGF WVSFEKREY
CYP9P24	MH500629	511	MEILVTCSAFTILAITLIAIILAKLITVIYRKQTYWKSRQVPYIRSVPILGVMWKFFFRRITILELSDEMYNSYPNARYV GFISAIFPAVLIRDPDLIRDVAVKYFDHFTDRYNFVNEEIEPMFGKSLSLLRGERWKEMRNILSPSFTASKMRFMF QLVSKCSQDFVDYLVEHPEYSSFIEAKDAFARYTNDVIFTVAFGINVNSLKDRENEYFVRGKTIFDDIFGSIFKVLII NTCPAFAKMIGMKIVSPATTSLFRRTISDALKTRREQGIVRPDMLHLLMQAKDKDSETELTVDDIVSQAFTFFLAG FDSSSTMMCFMVYELALNPDIQEKLRNEVDQYFDETNGEINYETLSKMEYMDMVVSETNRKHTFSVFIDRVCTQ EFELPPAEPGYNSVTIKPGDCVWFPDYALHHDPKYFPDPEKFDPERFNEENKGNILPYTYLPFGVGPRQCIGNRF ALMEIKILMVHLLRKFVIKPNEKTSVPLVFKKANFALVPVDGWWFSLEKRKF
CYP15A1	MH500638	494	MFYAVICLLLVLFCIFCVYDCFKPHNYPPGPKWMPLIGCFFTFRQLKLKNGYVYLAFHELMKSHGPILGLKLGTQK VVVISTHDLVKKVLLQDEFNGRPDGFFFRVRAFGKKRGILFAEGPTWAQCRRFTMRHLRAFGFGQSIMEKQLIIE AESFVDYLRRASAKGPVPMHTAFDVAVLNSLWSMLAGHRFDYDNEKLMEILDAVHGAFRLMDTMGGIVSHMPF LRFIIPELSGYNELMRILRKLWGFLDEEITIHEKQLSGNEPQDLIEAFLLEISSNSKIDDSIFDRENLLIVCLDLFLAGS KTTTDTLAATLLFLSLHSDWIKILQEELDNVVGRSRSPALKDSSSLPMMESFLAEVQRYLNLAPFGLPHKTMKDVN LNGYHIPKDTMILLDFHSVHNDKAYWDHPEEFRPQRFLDDTGRFCPNSASMIFSLGKRRCPGEMLARSTLFLFFT YVVYYFDIEISPDHGKPDPNGYDGFTISPKPYHLKLTLR

CYP18A1	MH500621	538	MFVEHAAQWAWKAMGGTRIEVLYTLLVFIGVLLVARCLQWLRYVRSLPPGPWGVPVFGYLPFLKGDVHLQYGE LAKKYGPMFSARLGTQLVVVLSDHRTIRDTFRREEFTGRPHTEFINILGGYGIINTEGDMWKEQRKFLHDKLRSF GMTYIGGGKKIMESRIMREVKTFLRGLASKGGRSTDVSASLGMSISNVICSLIMGVRFQHGDYRFKRFMDLIEEG FKLFGSMAAVNFIPVMRYLPCLQKIRNKISENRAEMADFFQKTVDQHRATFDESTVRDLVDAYLLEIEKAKGEGR ASLLFQGKNHDRQMQQILGDLFSAGMETVKTTLEWAIILMLHHPEAAAAVQEELDQVVGRSRMPALEDLPFLPIT EATILEVLRRSSVVPLGTTHATTRNVTLHGYTIPAGTQVVPLLHAIHMDPELWEKPDEFRPSRFLSAEGKVEKPEY FMPFGVGRRMCLGDVLARMELFLFFSSLMHTFELKSPQGSSLPSLRGNAGVTVTPDPFNVCLLPRNLDIIEDANN DTIFGGILRNIGSH
CYP301A1	MH500645	530	MNPRMNCHLQISMIRRLSQSQDILRRTLCSGTVTSATRDCTVDHDAAIQTRPYEEIPGPRPIPILGNTWRLFPVIG QYQISDMAKVSQIFHDEYGKIVRLTGLIGRPDLLFVYDADEIEKIYRQEGPTPFRPSMPCLVHYKSVVRKDFFGDL PGVVGVHGEPWREFRTRVQKPVLQPQTVRKYITPIEVVTSDFIKRMEEIKGEDGELPADFDNEIHKWALECIGRV ALDVRLGCLGGNLTPDSEPQKIIDGAKFALRNVAVLELKAPYWRYVPTPLWSRYVRNMNYFVEVCMKYIDAAVE RLKTKKSVNEADLSLIERILAKETDPKMAYILALDLILVGIDTISMAVCSILYQLATRPEEQEKVHQELVDILPDPSVP LTNSHLDRAVYTKAFIREVFRVYSTVIGNGRTLQNDTTISGYRVPKGVQVVFPTVITGNMEEYVTDAKTFKPERWL KQSSNEKIHPFASLPYGHGARMCLGRRFADLEMQVLLAKLIRSYKLEYHHEPLNYKVTFMYAPDGELKFKLLPR
CYP301B1	MH500624	352	MLQPQTARMYVNSIEEASTAFLKRIRKIRDKNDEVPDDFLNEIHKWSLESIASVALDVRLGCLDDDANIETQQLIDA VTTFFKNVGVLELKIPFWKLFSTPTWLKYVNDLDTIVRIMSKYTAAALSRIQEGKNSGKEASLLEKVLASENNTKLA TVLSLDLFLVGIDTTSSAVASTLYQLALHPEKQDLAYKEVCNIFPSKDTQIEGKHLEQLKYLKACIKETLRMYPVVIG NGRCMTKDTVIRGYRVPKGVQVVFQHYVISNLDKYFPRSKEYLPERWLQSDGVRHSFASLPFGYGRRMCLGRR FAELEMLVVISKILQYYKIEYHHEKLEYYINPMYTPKGPLNLKFIDR
CYP302A1	MH500633	506	MYRRLKKCSNGVIRKEVYVRFYSDNPGKCEIEGKLFKDIPGPRSLPIIGTLYKYLPLIGEYSFTKLYDSGRKKLNRF GPLVREEIVPNVNVVWVYRPEDIAEIFRAESGLHPERRSHLALLKYRKDRSDVYSTGGLLPTNGPEWWRLRKEF QKVTSKPQDIINYLEETNCVVQEFVELCNNERFEDFLEILSRLFLELTCLIVFDVRLNSFSEKERCKDSISSKLIEAA YTTNSAILKLDNGLQFWRFFDTPLYQKLRKAQTFMETIALELVSSKQKDMQARCCKKSFLTAYLENPALNIKDIVG MACDMLLAGIDTTTYSTAFALYHLARNPSTQDKLRSEAMQLLPECNQPVTADVLRNASYTKAVIKESLRLNPISVG IGRILQTDVNLNGYQVPKGTVVVTQNQIICRLPEYFNEPDTFIPERWLRENVDRKGKSVHPYVLLPFGHGPRSCIA RRFAEQNMQVLLLQICRRLKFSWHGDELGMISLLINKPSGPVKLSFDKI
CYP303A1	MH500649	561	MFSTVMLLVILLLLLLYLSSRKPKGYPPGPKWWPILGCAIEVARLRQKTGYLIKTCSALSKKYGPVVGLKIGTDRIV VLNDYESIRAMLTDEDCDGRPTGPVYQTRTFGTRKGLIVVDGNLWVEQRRFVLKHLRDFGFGRKSMATIIEEEA MSLVGHFKKLIDSDYNERINESKIHCNNNENVDGQIYKLIKKDNVDTSESTDKYITNKKHLTSSDKYMNMNDYVEI KKMDDGIVIPMHDAFGVTVLNTLWRMMAGKRFNLDDQDLTYLQQLLATLMNEIDMIGAPFGHFPILRFIAPEMSG YKSFLEVHQKLWKFLRDELDGHRNTFTPDSPRDLMDVYLDVLSSKNYSNTFSEAQLLAICVDLFMAGSETTSKSL

			GFCFLNLMLNPHVQKKAHEEIDRVIGRNRPPTLEDRSKMTYMNAIVLESLRIFMGRTLNVPHRALRDTSILGHRIP KDTMLIVNFDRILMGESWGDPENFRPERFINESGNIVTPQTYFPFSIGRHRCMGENLARSNIFIITTALLQAFTFSP VPGEEKPSSQDFVDGVTSGPKPFGALVSLRT
CYP305D1	MH500631	487	MFITVVLIILILILVVVKHKVKNQPPGPFPWPVIGNQFLLKRLTHKLGAQHFAFIELSKRYNSHLISLGSGANKVLVVS GSESCMMEVLKSEEFEGRPWNAFIEMRNMGKKQGITMNDGDEWREIRGWTMRTMRSFGFGKQNMLDMIKNE LNIILDKMKKGGVQRLKPMIVPAVLNVLWMLTTGKPFGDDRKLQYFTELLERRARAFDMNGGFLAAFPWLRYVA PETSGYNLLVTLNNELKAFLMDIINEHKLKYSPENNDDLIDLFLHEMYSENSSSSIFNEDQLVMVLIDFFLAGCTTT ATSMDFLFLIMVLHQDVQRKLQQEIDSVIPRDEFPELKHKIKLPYTEAVITESQRLWPPFPIIGPRRCLRDTNLGGY KIPKDSTILLNMYSIHVDPVLYPDPHTFNPERFIKDGVFEPNVNSLSFGKGRRRCPGEMLAKSAMFLLFTGIMQKY TLLPVPGKGPTSLEINPGLTISPKPYQSLVMPR
CYP306A1	MH500650	435	MGSVYTVLLSEPRLIKQTLAKDAFAGRAPLYLTHGIMQGYGLVCAEGERWRDQRKFVSSCLRNFGMVKHEGSR REKMEKRILDAADECVSVLEKRSANGPIDPLDTLHHYMGNFINSIVFGKTYQENEPVWKWLRHLQEEGVKEIGVA GPLNFLPFLRFLPRYGKTIRSIVEGKEKTHQIYRSILDQHRAEIAQSLPKENMTESFLAAFDEQMRKRGPVESAFY TEAQLYHLLADLFGAGTDTTLTTLRWFLLFMAAHPIEQEKIHLEMNRCLRKQEAPTLNDRSTMPRLEAALAEVQRI RSVTPLGMPHGTIEDSQIGDYYVPRGSMIIPMQWAVHTDPTYWTNPLEFQPDRFLTKDGNFFKPESFLPFQNGK RVCIGEELARMILFLFAGRILHAFVVSTPSDVDGVDLEGECGITLVPKPHRLIFVRRCDDA
CYP307B1	MH500620	506	MIPLTATTCFLIAVTFLALALILLDYLRSKKTSKNVIVDDDSHLLPEPPGPKPWPILGSLHILGRYNVPYKAFGDLVKE YDCEVIKLRMGSVPCVVVNGLENIKEVLITKGHHFDSRPNFDRYHLLFGGNKENSLAFCNWSEVQKTRREMVRA YTFPRAFSARYNELNGIINDEMKFLVDHLGSLSKKDVNTKPLILHCCANIFLTYLCSKNFRFEHAGFRNMVANFDR VFFEVNQGYAADFLPFLMPLHHRNMARMAHWSHAIRKFIIENIISEKLNNWNGMVPENNYMDCLINHVKTDAEPH MSWNTTLFVLEDIIGGHTAIGNLLVKVLGFLATRPRVQQIAQQEIDAVGITGHYVGLENRGSLPYVEAIILETVRIIAS PIVPHVANQDSSIAGYRIKKDTFIFLNNYALNMSNELWTSPEEFMPDRFVQNGRLLKPEHFLPFGGGRRSCMGY KLVQYLSFSIVATLLKNFTIVPVEKEDYTIPIGNLALPEITFKFRFERR
CYP314A1	MH500652	546	MLISSVWFEVIAAALLTTLIFATSYRPVWWFWSGASHRASAPAEVTDQDRRKFKTVSDVPGPYSLPIFGTRWIFS SIGSYTLNKIHDAYKATHRFVHFNATRFFLDSRIDLNQRYGALCKEEALWNFPVISVFSRQDIEAVIRRSPRYPLRP PQEVISHYRRNRHDRYTNLGLVNEQGQRWHDLRTALTSELTGANTVLGFFPALNVVADSFIDLIRRRRTMGYKV TGFEELAYKMGLESTCTLILGRHLGFLKQDSSSELSSRLAEAVRIHFTASRDAFYGLPLWKLLPTSAYKQLIESED AIYSIISDIVETTIWEKKNDAKDESVEAVFQSILKQKSLDVRDKKAAIVDFIAAGIHTLGNTLVFLFDLIGRNPKVQAKL YEESYSLAPPGCDLTTEDLRKAKYLRACITESFRMIPTTTCIARILDESLELSGYRLPAGTVILLHTWIAGLSEDNFK DASEYLPERWLTPIAPHSPLLVAPFGAGRRICPGKRFVELALQLILAKIIREFEIVVDEELGLQFEFILAPESPVTLGF RDRSQKA

CYP315A1	MH500609	530	MNCTRNVLKTVKSVSVVPVKRRIPDCGYAGASHSSRIDDLSDISKSADGAVKSKIEIAEKLRDRNYATAATTATEN VLQEAPEPWGFPVFGTIFSFLFFGGPKRQHEYVDKRHKELGPVYRERLGPVTAVFVNSPHEYLRIFRLEGSAPR HFLPEAWTLYNEIRKRRRGLLFMDGEEWVHFRKILNKVMLARNSTSLMAEPCYEVAKKFRQNWEKQIERNTIIED IQVQLYQWSIEAMLATLMGSSWHSCKEQLSRNEEKLAKTLYKIFEYSAKLSTMPAKLAMALRLPAWTKFAESADT AFEIVRILVPEMTRLGGNGLFKKMLDEGIQEEDAICIVTDFILAAGDTTATTLQWILLLLCKYPDMQEQLFEHLKDLP EKELLRDSLLKGVIKEALRLYPTAPFISRYVPEDSVIGNYFVPKGELVVLSLYSSGRDSKNFPQPNEFLPERWIRTE SGTYKGVIHSHASLPFALGARSCIGRKLAEIQISLVLAELIKSFKIDCINKDEVKLILHLISVPSESIKLKLTRR
CYP334A1	MH500647	570	MKLSKPLATNDSQKCLDLNASDILSAPITQSQTTSVMNESLTRTNSIPLLDQSAAEVSTATFEITTSKNESQIIGDRS AQPFEEIPGPAVLKIWEKYWKFVPLFGTQLFSSLLINRFTQGRLSWNRNVTPLKYLFNEYGCIVRINGPLSGDIVMI HRPEHIAEVFKQEGDTPLRSGIDILQHYRLNYRKYRLAGAFSLQGSEWLELREKVEQEFEQIASNFFDRIDAICDE LINRIYKIRNRQNEVPATFHEDLLRWGMECFCNLTFNKHLGFLESAGYNSTSETSRIVNAMMVAHKYMSRCETGF QVWRFFLTPFARKLFDACDVLDGVIGKYVRHAQNKLRSRSPQDVEGGSPVLEKFLLNEGIRPDDLCTLLMDMMIL GVQATVNSEAFLLYYLARNPRTQRKIYDEIVSVLPSSDSPFTEKTVRNMPYLKACLQESLRLRPAVPYITRLLPKTI SLHGYTIPKGTFVIMANQITSQREENFEDPEKFRPERWLNSKKEDTDFSYLPFGYGARSCLGKNMAEAKMMLLT AKLIRQFRIEYDYADIKSSFMMVNVPNKPLRFRFVDRN
CYP336A35	MH500619	501	MASACFTLVIGALVLLTLYLIKKYTYWKRRGIPTVRGIVPFVGNILPVLTMKYNFFDYNTKLYNDYKNYSMIGYYKLL KPVLLIRDPELVKTVMQSKFSNFHDTGISLRPELDPLLSKDPFFASGDEWSRGRKKLTYAFSNTKLKVFYAAVSG VCKKFEDFLIRQLTDNEKYEIELKQLFSKFTAEIVANAGFGLEGFSFQEKKKPEAFEAISQTMSDFSFFRGFINNIVF IPEIRNLLRLRLLPREFDQLFRNIVKESLEIRRNDPTPKNDYLQLMMDLSKSTNEPISDEDTAAGALSFYVNGFETS GNTLSFVSYYLATHPDIQEKLRQEITSKIADYEGVLTFEALKEMTYMDQVISESLRCYPVISSFSRVCTEAYELQGP DGLKCRVEPGTQVIIPVHALQVDPQYWSDPEVFDPERFNEDRKKSIVKMTFLPFGEGPRMCVGMKMGMLQVKS CLATLLNKYKLELSNKTQLPLKLSPYAFVTVPIGGLWVHISKI
CYP336A36	MH500617	501	MASTFFTLLVGAVLLFILYFINKYTYWKRRGVPTVKGVVPFAGNILPVLTLQSNFNDMHTKMYKDYKQYSMVGYY KLWTPVLIVREPQMVKTILQSNFSSFHKNALTVREDLDPLLVKNPFFTSGESWSRSRKRMTYAFSNVRLKSLFVT VNGVCKKFEDFLNRRLAFDDKYETELKHLFSKFTGEVVANAGLGIEGFCFEDTKHAGAFDIISEPIFEPSFFRAFE NNALFITELRNLLRSKWIPKEFDAFFRNIVKENIEIRQKESTPRNDFLQVMIDLMKTSNENIDMEAITAEALSFYVDG FETSSITLSFIGYNLAAHPDVQEKLRQEITSKIAKYDGVLTFDALKEMTYMEQVMNESQRLYPALSALSKICTEKFE LQGSDGLTLQVEPGTEIIIPHHGFQLDPQYWVDPEVFDPERFNEEKKQSIVKMTFLPFGEGPRMCVGMKMAMLQ MKACLATLVGKYKFTLSPKTQIPLKMTPYHFLAAAQGGLWVHISKI
CYP336L1	MH500626	502	MAFAFVILSVIILILLMFFLIKKYTYWKRQGIPTVRGTLPFVGHMLPVLTTKLNYSELNRKMHIEYKNHSMVGYYKLT NPVLLIRDPYLAGIVLKSKFSCFQRTGVVAHPDIDPLLARNAFFVAGEEWTNARRRLINSFTNVKLKTLLESINVVC KKMEDYIDRRLQTTKKYETELKTLCSKFTGEVIANVGVGVEGYCFDEKPHPAAFDRIGDWLFKSNLKQVLEMTLM

			FTPGLKNSMLKMKFIPEKMESFFRDIVSQNLKIRRQDSIPRSDILQSILDYLNWEKEEIDEELIASDVLSFYMDGYET SAVAISFTAFLLAQYPEVQDKLRREIMSKIEKYDGAVTFEGLREMTYMDQVINETQRRYTSISFLTRVCTEECELR GSDGLIYRVEPGMEVIVSNQGFHLDPEYWPDPELFDPERFSEERKHTIEKLAFLPFGEGPRLCAGMKMAQLQLK SFLTTLISKYRLELSEKTQLPIKLSGLNFLTEPIGGLWVHISKI
CYP343A1	MH500634	440	MYGPVVGLRLGFDEPLIIVSGKAAITEMLHRSEFDGRPNGFLFKYRSGGTRQGLLFTDTNVWHSQRRFTLKTLKE FGFGKQIMEHVLQEDAIMLTNIVTELTKTGTPTSIQSIISAAVLSNLWLLIDGTKFDVGMESPMLKEAINILKDLTSSS NVMGGILNQFPFLRYLFPNLTGFSVFAERQSRINSFFMNIVSKHKETMNETKHECTNFIDAYLQEIETRKTSSSSS FFNENQLAYVVKDLFAAGVDTTDNTIGFVIAFLIVHQDVQMKMHEEIDRVIGRDTYPSLNDCNRLPYLKAILSEVW RLANIGPTSIPHRAIIDSNLLGFEIKKNYTLLANLKSVHMDKEHWGDSEVFRPERFIDSKGQFVEDSWLMPFGLGR RKCLGETVARQTTYLFVACLLQRLHFKLPLNHPKPRLQGTEGFVVMPPKLDIIAVQRY
CYP369A1	MH500636	506`	MSALLIVILLLIIAYKFYEFLSCIPPNAPPCIPRLPVGGSYWYLLWGDYKFPHKTVSYYVKKFQSKILSCYLGGFMAV IANDYESIKEILTREEFDGRATDVDVVKARAFGKELGIFFTEGSFWKEQRRFALRHMRDFGFGRRHDRFENDMM EELNILVDMLKEGPINDDEKSYLKHGYASFPNILYPYSANSIWDIMFGDRFNRSQHQKLIYLCQAAMKFQRGGDT TGAAIFQRWFLKYFGDMFGYKSIIDGSYRIASFVMKHIEERKQLYDEDIDGRGLIDRYLSELKKNSNVMSTFSEEQ LIILLVDIMFPALSAAPSAIVHAIKLLMHNPNVLKNAQEEIDRVVGTGRHVTWEDRKNMPYTEAVIREALRYETLTPF GVIHKTIKDTTLGGFNVPKDTLAITNLDELNHDPDLWGDPENFRPERFLAEDGQLGKDFTFVFGLGHRVCAGETF ARYNMFGVLAVLIQNFHFSFVEGQPTSLEDKLPGLITTPKETWIKVEPRY
CYP9BU1	MH500604	517	MEYLTITLSVVAILTAIYYYYLKNLNVFKKYGIPHIPPKPIVGNFGSMMTMQSSILDIVQKVYDLCPDAKYAGMYEFT SATILLRDLDLIKSICVKNFDSFQDHRGFLDKKADPLFAGVLFFLDSEEWKSQRNLVSPVFTSSKIKTMFKLMSAC AVRFADYLSKLPESEREFELKSVLSKFTNDVIATCAFGIEVDTIKDPNNELFVKGKQISNIGSFTNLMKQMMNRNM PFLANMLNLPFLPRNITDFFEDLIIDTMKTRQEKGIYRPDMLQLLMDQSNKKEGKGLTNFNIASHAFGFYFGGFDS VATQTCFVFQMLAENPDVQAKLQAEIDEVLENNNRQLTYEAISGMTYLDAVVNETMRLYGISFVLDRVSTKAFEL PPAVPGAKPFTVQPRTNIWIPASAIHRDPKYYENPDKFEPERFVNDKSIINSGAFMPFGVGPRMCIGNRFALTKM KVIICHILARCEVKLSPKSRVPLEMSTSSFALQPKNGFLLRVEPRKNIPTVNVDWINGVSQ
CYP9BU2	MH500605	509	MEYLTIALSVTAVLIACYYYYLKNLNVLKRYGIPHIPPTPIVGNLGPVLAMKCSMMEMIKKVYNIDSEAKYVGFYDL GVPMIFIRDVELLKTIGMKNFDHFQDHQGFFDTSVDTLFGGSLFFLHGDYWKNQRSTVTPIFTSSKIKNMFKIMTD CGERITDYLLKLPEEEREIEMKSVLSKYTNDVIASCAFGIEVDSVKDPNNELYVNGQRTMLFVGFGQFIKILMYRN VPSLIKLLRIRFFAKNLADFFQNLLLDTVKTREEKGIYRPDMLQVMIDAKNKNEPGKTLTISEVISNAYGFYFGAYET VAMQSSFVFHLLAEHPDVQRKLQEEIDKVLEENDGQLTYDAVMKMEYLEAVIYETMRLYPNAFFLDRVCTKTYEL PPAVPGAKPYTLRPGMTLWIPVCAIHTDSKHYENPDKFDPERFVTDGKRILNSGAFIPFGFGPRMCIGHRFAMTK MKVIVCQTLSRCQFKLASKSQLPLEILKGSFAAIPKNGFRLKVEPRKNVNLN

CYP9R1	MH500608	517	MWTIILAVVAVLVVVYKYMTDYDFFEKRGIPYAKPFPFLGSIWEAVLMRCTFAEVVRQLYELNREAKYVGFFDFLT PVVTIRDLDLIRSVTIKNFDNFPDHRSFQNDEIDPLFSKNLFNLRGDRWREVRTTLSPAFTSSKMKAMFILMRECA KKYGENLSSLPADQRILELKDAYTRYTNDVIATCAFGVNVDSMTDRKNKFYVYGREATTFGRLQSLKFFLIRSFPK LCNLLNVKIVKTEIAEFFQNLVSDTIKVRDERGIVRPDMIQLMMESRGKMGPGKELTIEDMTAQAFIFFFGGFESTS TTMCFATYEVGVNPDVQKKLQEEIDQVLEDCNGEVTYEAINNMKYLDAIINESLRMYPVIVAVDRICMKDFELPPA LPSVKPHVVKKGEYIWIPIYGVQHDPDHFPEPDKFNPDRFLDDPKKIMNSGAFLSFGLGPRMCIGNRFALLEAKTL LFHVFANCELKPCSKTTIPMKLSKQGFAMTADGGFWFDVQPRKKPQHVMVNSVSSNGVAF
CYP9R38	MH500606	517	MWTIILAVVAVLVVVYKYMTDYNFFEKRGIPYDKPLPLFGSVGQAVLMRCTFAEVVRQIYEFNREAKYVGFFDFLT PVVSIRDLDLIRSVTIKNFDNFPDHRSFQDNEIDPLFSKNLFNLRGDRWRDVRTTLSPAFTSSKMKAMFILMKECA KKYGENLSSLPADQRILELKDAYTRYTNDVIATCAFGVNVDSMTDRKNKFYVYGREATTFGRLQSLKFFLIRSFPK LCNLLNVKIVKTEIAEFFQNLVSDTIKVRDERGIVRPDMIQLMMESRGKMGPGKELTIEDMTAQAFIFFFGGFESTS TTMCFATYEVGVNPDVQKRLQEEIDQVLEDCNGEVTYEAINNMKYLDAIINESLRMYPVVVATDRICMKDFELPPT LPSAKPYVVKKGEYIWIPVYGVQHDPDHFPEPDKFNPDRFLDDPKKIMNSGAFLSFGLGPRMCIGNRFALLEAKT LLFHVFANCELKPCSKTTIPMKLSKQGFAMTADGGFWFDVQPRKKPQHVMVNSVSSNGVAF
CYP9R39	MH500607	516	MIWTIIVASVVVLAIYHFTSKRYAYFEERGIPYLKAFPFLGSLWKVVLMRASFAATVQQMYNLYSEAKYVGCFDFL TPVILLRDPELIKSVMIKNFDHFPEHRNLSDNADDTLFSKNLFSLNGERWKEVRSMLSPAFTTSKMRSMFVLMKE CAKRYGENLASLQADQTILELKDTFTRYTNDVIATCAFGVSVDSMTYRENKFYLYGREASTFGLKQSLKFFFIRSF PRLCRMLKITLLRKGVSDFFKDLIESTIKTRDEQGIVRPDMLQLMMEFRDKGDPSKELTLDDMIAQAFVFFLAGFE STATLMCFTTHEVGVNEEVQKRLQDEIDQVLTDCNGNVTYEAINGMKYLDAIVNEGLRKYPVSVAGDRVCRKPF ELPPTLPNKKPYVVKEGEIVSIPFYGIQHDPKYYPEPEKFDPDRFYDDPKQILNSGTFLTFGLGPRMCIGNRFAILE TKTLLFYVFANCTLKRCSKTIVPMKLSKQGLAMTPEGGGYWFEIQPRAKGQSLIANGDNDRK

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